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Research Proposal:

ADHERENCE OF RAG-1 TO HYDROCARBON AND HYDROPHOBIC SURFACES DURING GROWTH

Submitted To:

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July, 1979

INTRODUCTION

The adherence of bacteria to a wide variety of inorganic surfaces has been well established, both in natural environments and in the laboratory (1 - 4). The advantage gained by "sticky" bacteria is perhaps most evident under circumstances in which the cells are able to adhere to surfaces which can provide necessary nutritional sources for metabolism and growth.

One interesting and important example of this phenomenon is the growth of hydrocarbon utilizing microorganisms on the surfaces of insoluble liquid and solid hydrocarbon (5). Growth of bacteria and yeast on hydrocarbon as a source of carbon and energy has received increasing attention in recent years. Potential applications are sought in such varied fields as food technology (6) and ecology (7). In addition to providing an inexpensive source of single cell protein, hydrocarbon degrading microorganisms and their extracellular products may provide a biological solution to oil pollution problems.

The adherence of bacteria to hydrocarbon may be viewed as one example of the more general phenomenon of interactions between bacteria and non-wettable, hydrophobic surfaces.
Other examples include the adherence of sulfur-oxidizing bacteria to insoluble sulfur particles (8,9) and the attachment of marine bacteria to a variety of hydrophobic surfaces (10-12).

Studies on the growth of microorganisms through direct contact with the bulk hydrocarbon phase are generally complicated by emulsion formation during growth (7). Many hydrocarbon degrading yeast and bacteria release emulsifying agents which "pseudo-solubilize" the hydrocarbon phase in the form of microscopic droplets while the culture is being mixed. Reports on the effects of endogenous production or exogenous addition of emulsifying agents on the degree of direct contact between the cells and the bulk hydrocarbon phase are often ambiguous and sometimes contradictory (13 - 18). A better understanding of the relationship between emulsifier synthesis and direct contact between the cells and the hydrocarbon phase is thus necessary.

The more general problem of how wettable bacteria are able to attach and adhere to non-wettable surfaces is to-date somewhat enigmatic. A better understanding of this general phenomenon may contribute to our ability to combat various problems, such as those related to marine biofouling (10).

In the proposed research, the characteristics and mechanism of adherence of the marine bacteria, RAG-1 to insoluble alkanes during growth will be studied. RAG-1 grows on higher alkanes as well as various non-hydrocarbon sources of carbon and energy (19). During growth on ethanol, acetate and hexadecane, RAG-1 synthesizes and releases a viscous, acidic polysaccharide of high molecular weight (about 10^6) which has emulsifying properties (20 - 22). This macromolecule, termed emulsan, differs from other emulsifying agents which have been isolated from hydrocarbon degrading microorganisms in its large size, its structure, and the fact that it is a relatively poor surfactant. The physical and chemical properties of emulsan are currently under investigation in this laboratory.

MAJOR GOALS

It is intended that the proposed research will:

1) contribute to our understanding of adherence phenomena involving bacteria and hydrophobic surfaces;
2) examine the role of the outer cell surface in the adherence of RAG-1 to the bulk hydrocarbon phase;
3) elucidate the dependence of bacterial adherence on metabolism in the system to be studied;
4) generate a model which will account for the attachment and growth of bacteria on the bulk hydrocarbon phase.

PRELIMINARY WORKING MODEL: FACTS AND HYPOTHESES

1) The marine bacteria UP-2, has been observed to adhere to and grow on both liquid (hexadecane) and solid (tetracosane) n-alkanes through direct contact with the bulk hydrocarbon phase (5, 19). RAG-1 has also been observed to adhere to solid and liquid n-alkanes during growth on these substrates.

2) During growth of RAG-1 on hydrocarbon in the absence of mechanical agitation, the great majority of the cells will grow through direct contact with the bulk hydrocarbon phase.

3) Antibodies raised against emulsan cross-react with wild-type RAG-1 cells. This indicates that emulsan is present on the exposed cell surface prior to its release.

4) Emulsan contains fatty acid residues which are covalently bound to the sugar backbone (23). The fatty-acid composition of emulsan has been shown to be dependent upon the carbon and energy substrate on which the cells are grown.

It is postulated that cell-bound emulsan plays a decisive role in the process by which RAG-1 is able to adhere to the hydrocarbon surface. It is further suggested that emulsan-bound fatty-acid residues may be exposed at the outer cell surface, thereby increasing the hydrophobicity of the cell exterior, and enhancing the cell's ability to bind to the hydrophobic surface. Fatty-acid composition of the cell surface has been shown to have an effect on the ability of yeast cells to bind emulsified hydrocarbon (24, 25).

5) It is possible to show that the adherence of RAG-1 to the hydrocarbon surface is a time-dependent process, and that its attainment requires cellular metabolism. It should also be possible to show that once the cells adhere, direct contact need not be maintained by the expenditure of cellular energy.

6) Emulsan synthesis has been shown to be dependent to a certain extent on growth conditions. It is thus assumed that emulsan synthesis is subject to cellular regulation and can be inhibited under certain conditions.

7) Microscopic observation of adherent cells of both RAG-1 and UP-2 during growth on solid hydrocarbon reveals that the cells are not evenly distributed over the exposed hydrocarbon surface, but are concentrated in densely colonized patches, leaving other surfaces relatively bare.

It is postulated that the dense patches are microcolonies, i.e., the progeny of single cells which grew and divided without ever leaving the hydrocarbon-water interface.

PREDICTIONS AND EXPERIMENTAL APPROACH

1a. Conditions which repress or inhibit emulsan synthesis should also lower the degree of adherence.

Experimental - An accurate quantitative assay for cells bound to solid, bulk hydrocarbon has recently been developed (5). In addition, efforts will be made to develop a simpler method for enumerating bound cells. In the envisioned system, the hydrocarbon substrate will be evenly adsorbed to a membrane filter, which will be suspended in liquid medium. At any given time, one or a number of such filters can be removed from the aqueous medium, washed, clarified, and the bacteria bound to the filters counted either visually or using existing fluorescent staining techniques (26, 27). The number of cells in the aqueous phase can be determined by viable counts.

The concentration of emulsan in the medium can be quantitatively determined by an existing assay (21).

The effect of growth conditions on adherence and emulsan synthesis will be studied for parameters known to have an effect on polysaccharide synthesis by bacteria (28 - 32): nitrogen, phosphate, and sulfate concentration, and growth temperature.

1b. Adherence should be inhibited by hydrolysis of exposed fatty acid esters.

Experimental - Cells will be exposed to brief alkaline hydrolysis as well as treatment with esterase, in order to remove exposed fatty-acid residues. Adherence will be assayed as described above.

1c. Inhibition of de novo synthesis of fatty-acids should reduce the fatty-acid content of emulsan and inhibit adherence phenomena.

Experimental - The exogenous addition of fatty-acyl-coenzyme A has been shown to inhibit de novo synthesis in bacteria, without affecting cell viability (33). The effect of various concentrations of hexadecanoyl-Coenzyme A on the adherence of RAG-1 to non-metabolizable hydrophobic surfaces will be tested during growth on soluble carbon and energy sources (see also section 3).

2. Following transfer of cells from conditions which do not favour adherence to conditions which do favour bacterial attachment, a lag should be observed. The addition of inhibitors of oxidative phosphorylation should inhibit adherence when added during the lag period but should not desorb cells which have already become attached.

Experimental - Inhibitors such as cyanide and azide will be added at various times following the transfer of cells and the degree of adherence will be measured.

3. Hydrophobic non-metabolizable solid surfaces may be substituted for the metabolizable hydrocarbon surface as substrata to which the bacteria adhere. Such surfaces include various polymers as well as hydrocarbons which the bacteria cannot degrade. Cells may thus be "tricked" into adhering to such surfaces as if they were metabolizable. Such a model system will enable us to study the effect of soluble carbon and energy sources on adherence phenomena.

Experimental - The experimental procedure will be that of Fletcher and Loeb (11), with the major exception that growth media will be employed, in addition to buffers.

4. Mutants deficient in their ability to adhere to hydrophobic surfaces can be isolated. Such mutants may not be able to grow in the presence of bulk hydrocarbon phase, as the sole carbon and energy source, but may be able to grow on finely emulsified hydrocarbon. It will be interesting to see whether:
    a) adherence-deficient mutants are also deficient in emulsan synthesis;
    b) mutants deficient in emulsan synthesis (certain classes of these mutants have already been isolated) are also deficient in their adherence ability;
    c) the non-adherence phenomena of the mutants can be reversed by the exogenous addition of emulsan.

Experimental - Mutants deficient in their ability to adhere to the hydrocarbon surface during growth will be isolated in the following manner: Mutagenized cells will be grown in a medium containing sufficient concentration of soluble carbon and energy substrate to permit growth yet not affect adherence ability. To this medium either toxic hydrocarbon or hydrocarbon containing a paraffin-soluble, water-insoluble bacterial toxin will be added. The assumption is that adherent cells will come into contact with very high concentrations of the toxin and be selectively killed, while non-adherent cells will survive.

5. Clusters of bacteria observed on the hydrocarbon surface (see page 5) are the descendents of a solitary, adherent cell.

Experimental- One way to test this hypothesis is through the use of a mixed culture. RAG-1 and UP-2, for example, can be grown on the same medium on the same hydrocarbon source. Since these two strains can be distinguished visually, the clusters will be observed to be either homogeneous or heterogeneous.

A more rigorous approach involves the use of a mixture of RAG-1 wild type and auxotroph (for amino-acid, for example), grown on hydrocarbon adsorbed to a membrane filter. After sufficient time is allowed for development of clusters, the filter paper is replicated onto selective plates which can distinguish the wild type from the auxotroph. In this manner the degree of homogeneity of the clusters can be ascertained.

REFERENCES

1. Costerton, J.W., G.G.Geesey, and K.-J. Cheng. 1978. Sci. Am. 238, 86.
2. Fletcher, M. 1977. In: Microbiology (D. Schlessinger, ed.) A. S. M., Washington.
3. Gibbons, R.J.. 1977. In: Microbiology (D. Schlessinger, ed.), A. S. M., Washington, p. 395.
4. ZoBell, C. E. 1943. J. Bacteriol. 46, 39.
5. Zilber, I. 1979. Ph.D. Thesis, Tel-Aviv University.
6. Johnson, M.J. 1967. Science 155, 1515.
7. Gutnick, D.L. and E. Rosenberg. 1977. Ann. Rev. Microbiol. 31, 379.
8. Schaeffer, W.I., P.E. Holbert, W.W. Umbreit. 1963. J. Bacteriol. 85, 137.
9. Schaeffer, W.I. and Umbreit, W.W. 1963. J. Bacteriol. 85, 492.
10. Dexter, S.C., J.D. Sullivan, Jr., J. Williams III, and S.W. Watson. 1975. Appl. Microbiol. 30, 298.
11. Fletcher, M. and G.I. Loeb. 1979. Appl. and Environ. Microbiol. 37, 67.
12. Fletcher, M. 1977. Can. J. Microbiol. 23, 1.
13. Aiba, S., V. Moritz, S. Someya, and K.L. Haung. 1969. J. Ferm. Tech. 47, 203.
14. Borzani, W., B.R.V. Concone, M.L.R. Vairo, P.A. Doin and J. Kovaks. 1971. Recent Advances in Microbiology, X Int. Congress, Mexico City, p. 419.
15. Chakravarty, M., P.M. Amin, H.D. Singh, J.N. Baruah and M.S. Iyengar. 1972. Biotech. Bioeng. 14, 61.
16. Miura, Y., M. Ozaki, S.-I. Hamada, S.-I. Murakawa and R. Yugan. 1977. Biotech. Bioeng. 19, 701.
17. Mimura, A., S. Watanabe and I. Takeda. 1971. J. Ferm. Tech. 49, 255.
18. Velankar, S.K., S.M. Barnett, C.W. Houston and A.R. Thompson. 1975. Biotech. Bioeng. 17, 241.
19. Horowitz, A., D. Gutnick and E. Rosenberg. 1975. Appl. Microbiol. 30, 10.

20. Reisfeld, A., E. Rosenberg, and D. Gutnick. 1972. Appl. Microbiol. 24, 363.

21. Rosenberg, E., A. Zuckerberg, C. Rubinowitz and D.L. Gutnick. 1979. Appl. and Environ. Microbiol. 37, 402.

22. Zuckerberg, A., A. Diver, Z. Peeri, D.L. Gutnick and E. Rosenberg. 1979. Appl. and Environ. Microbiol. 37, 414.

23. Belsky, I, D.L. Gutnick and E. Rosenberg. June 1979. FEBS letters.

24. Kappeli, O. and A. Fiechter. 1977. J. Bacteriol. 131, 917.

25. Kappeli, O. and A. Fiechter. 1976. Biotech. Bioeng. 18, 967.

26. Watson, S.W., T.J. Novitsky, H.L. Quinby and F.W. Valoris. 1977. Appl. and Environ. Microbiol. 33, 940.

27. Snellen, J.E., A.G. Marr and M. Starr. 1978. Current Microbiol. 1, 117.

28. Norval, M. and I.W. Sutherland. 1969. Jour. Gen. Microbiol. 57, 369.

29. Heptinstall, S., A.R. Archibald and J. Baddiley. 1970. Nature 225, 519.

30. Duguid, J.P. and J.F. Wilkinson. 1953. Jour. Gen. Microbiol. 9, 174.

31. Markovitz, A. and S. Sylvan. 1962. J. Bacteriol. 83, 483.

32. Markovitz, A. 1977. In: Surface Carbohydrates of the Prokaryotic Cell (I. Sutherland, ed.). Academic Press, London.

33. Ascenzi, J.M. and J.R. Vestal. 1979. J. Bacteriol. 137, 384.

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SPECIFIC INTERACTIONS IN BACTERIAL COLONIZATION OF TEETH AS A POSSIBLE TARGET FOR PREVENTION OF DENTAL CARIES

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March, 1979

ABSTRACT

The colonization of oral bacteria on the tooth surface and their ensuing metabolism has a direct effect on dental cariogenesis. This colonization may be achieved in part by specific interactions which promote the bacteria's attachment and adherence to the tooth surface.

It is the purpose of this paper to examine some of the evidence in favour of the role of specific interactions in the development of dental plaque, and to suggest methods of prophylaxis aimed at interfering with these interactions.

INTRODUCTION

Bacterial Adherence-

The adherence of bacteria, both to inorganic substances, such as rocks and glass, and organic matter, including a wide range of host tissues, has been well established [6,14,15]. Bacterial attachment often imparts such 'colonizing' cells with distinct ecological advantages. We may thus infer that the various modes of attachment are at least partially the result of bacterial adaptation and selection.

Bacteria may adhere to surfaces by general interactions, such as gross physical entrapment[14], or general electrostatic attraction[28]. They may also adhere by binding to particular molecules or molecular residues which they specifically recognize. While _in situ_ bacterial adherence to teeth probably involves a combination of general and specific interactions, this report will concentrate on the latter type.

The Nature of Dental Caries-

A direct relationship exists between oral bacteria and dental caries [29]. A high carbohydrate diet which leads to a high incidence of dental caries in laboratory animals raised in a normal environment does not induce caries formation in laboratory animals reared in a bacteria-free environment [39].

A similar causal relationship exists between cariogenicity and dental plaque [15]. Dental plaque is a general term for the organic matter which accumulates on the tooth surface, and may contain up

SPECIFIC INTERACTIONS INVOLVED IN PLAQUE FORMATION

The cariogenic virulence of oral bacteria is related to their ability to adhere to and form part of the dental plaque [14].

Attachment of bacteria to the plaque and their subsequent adherence may depend on a variety of specific interactions: between bacteria and adsorbed salivary proteins [29], between bacteria and the insoluble polysaccharides which they synthesize [15], and direct interactions among the cells themselves [28].

Cell-Cell Interactions -

Visual proof of specific interactions which occur between different bacterial strains within the plaque is obtained in scanning electron micrographs [23]; these show specific three-dimensional arrangements in which cocci are bound to the surface of filamentous bacteria in a highly-organized aggregation resembling a corn cob.

Cells of Streptococcus mutans in plaque are probably bound together in aggregates via the dextran molecules which they synthesize [15]. This phenomenon will be discussed later.

Cell-Saliva Interactions -

Salivary glycoproteins are able to aggregate a variety of oral bacteria in vitro [21]. What makes this phenomenon particularly interesting is its selective nature - two different strains, S. sanguis and S. mitis are aggregated by different salivary constitutents [25]. In another study, it was demonstrated that oral streptococci selectively bind salivary glycoproteins which comprise part of the acquired pellicle [41].

Bacterial aggregation by saliva may be mediated by lectins which are present in saliva [6]. Concanavalin A (Con A), a lectin isolated from the jack bean plant, greatly enhances aggregation of several oral streptococci in the presence of saliva [26].

Specific interactions between saliva and oral bacteria may be interpreted as interactions which enable the cells to bind to salivary proteins adsorbed to the plaque [15]. However, there are those who view these interactions as mechanisms by which saliva is able to remove bacteria from the oral cavity. One theory has it that the glycoproteins in salivary excretions mimic receptors present on epithelial cells, thus reducing their adherence to host tissue [43].

Streptococcus Mutans: Cariogenic Guinea Pig -

Most recent studies on dental plaque are carried out in in vitro systems which cannot duplicate the situation in the mouth, yet are more amenable to controlled experimentation. Whereas dental plaque contains quite a heterogeneous microbial population, one particular oral species is most frequently chosen for laboratory study - S. mutans. This choice is not capricious - various studies have shown that S. mutans is directly involved in cariogenesis, and that its cariogenic capacity is related to its ability to form adherent plaque [8,22].

The ability of S. mutans to colonize smooth surfaces and initiate plaque formation is primarily dependent on its synthesis of adherent insoluble polysaccharide molecules (sometimes termed glucans, but usually referred to simply as dextran, as they closely resemble classical dextran[15]), from sucrose. [16,35,36] The enzyme responsible for dextran synthesis, dextransucrase (sometimes referred to as glucosyl transferase) adds glucosyl residues to non-reducing termini of dextran molecules [5]. Dextran is able to bind the enzyme, forming high molecular weight, salt-insoluble aggregates (up to 150 enzyme molecules may bind to a single dextran molecule of molecular weight $2x10^{6}$) [13]. During early stages of growth of S. mutans in a medium containing sucrose, much of the enzyme activity is recovered in the supernatant; as growth continues, more and more of the enzyme activity is found to be cell-associated [7,20,34] The presence of cell-associated enzyme promotes the dextran binding capacity of the cell [42].

It is not surprising that adherence of S. mutans to smooth surfaces correlates with dextransucrase activity [30]. What is of particular interest is that it appears that the cell-associated enzyme does not bind directly to the cell but rather to dextran molecules which are themselves cell-associated. The following observations support this premise:

1. Dextranase eliminates the ability of dextransucrase to bind to the cell [34] .
2. Adsorption of dextransucrase to the cell is inhibited by anti-dextran globulin [20] .
3. Soluble glucan interferes with adsorption of the enzyme to the cell as well [20,34].

These and other observations have led Germaine and Schachtele[13] to propose a model for the specific interactions of adherence between S. mutans cells, the dextran which they produce, and the enzyme, dextransucrase (see figure 1).

The main features of the model are:
1. Dextran binds to a specific protein (or glycoprotein) on the cell surface.
2. Dextransucrase participates in the cross-linking of cells through

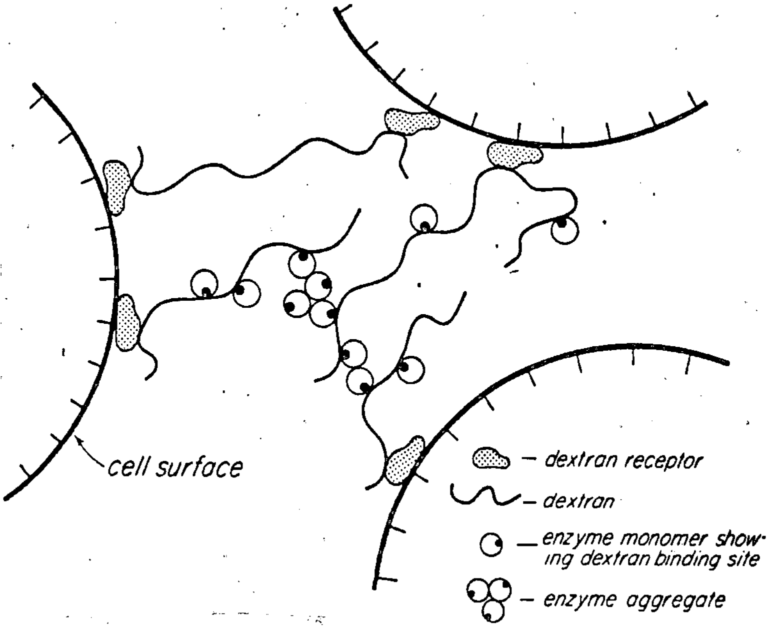
cell surface
– dextran receptor
– dextran
– enzyme monomer showing dextran binding site
– enzyme aggregate

Figure 1 - Schematic representation of the association of dextransucrase with the cell and its participation in cellular aggregation. Direct dextran cross-linking of cells through dextran receptor proteins is also depicted. From Germaine and Schachtele [13].

its association with dextran molecules which are cell-bound. This implies that the enzyme has either multiple dextran binding sites, or exists as an aggregate of enzyme molecules.

3. In some cases, single dextran molecules may cross-link cells.

Additional evidence for such a model comes from the study of mutants of *S. mutans* which produce only extracellular enzyme [7]. Addition of either sucrose or dextran to the cell medium does not cause the dextransucrase to become cell-associated. This implies that the mutation eliminated recognition between the cell surface and dextran molecules.

S. sanguis cells are also able to bind the cell-free dextransucrase which is synthesized by *S. mutans*, can synthesize their own cell-bound glucans, and exhibit adherence phenomena in the presence of sucrose [20,30]. These phenomena are thus not limited to a single strain of oral bacteria.

Another phenomenon which may play a part in the attachment of cells to plaque, and perhaps their detachment therefrom, as well, is that of cellular aggregation[15]. In one study of this type the ability of dextran to aggregate *S. mutans* cells grown in various growth media was examined [42]. Following growth, cells were washed and incubated with externally-added dextran. Cells grown in the presence of sucrose are readily agglutinated (by dextran concentrations as low as 5 ng per ml), while cells grown in the absence of sucrose showed little or no agglutination. Aggregation by dextran is prevented by pretreating the cells with proteolysis or heat[27].

Surprisingly enough, the phenomena of cellular adherence and aggregation of S. mutans in the presence of dextran appear to be different processes, involving two separate mechanisms. Isolated mutants of S. mutans which are unable to form adhesive plaque can still be aggregated upon addition of sucrose or dextran[12]. Moreover, certain inhibitors of dextransucrase activity (such as maltose) do not affect the aggregation process[16].

R.J. Gibbons has proposed that the overall adherence and colonization of S. mutans involves at least three distinct interactions of a specific nature: cell-dextran binding, dextransucrase association with its product, dextran, and cell attachment to salivary glycoproteins[11,17]. More work will have to be done before the precise nature of these mechanisms and the degree of their mutual interdependence can be ascertained.

PROPHYLAXIS

Can our limited present knowledge concerning the specific processes of recognition involved in the attachment of oral bacteria to the tooth and their subsequent proliferation on its surface be of value in preventing tooth decay? At least two general possibilities are worthy of consideration: competitive inhibition of such recognition processes by small sugar molecules which can compete for the recognition sites, and competition for the specific binding sites by addition of proteins which have high affinity for the same specific sugar residues. These possibilities will now be discussed.

Lectins and Dental Caries -

In the last decades the tremendous amount of research which has probed into cell recognition has shown that such interactions are often mediated by recognition of specific sugar residues, exposed upon the cell surface.

A major class of proteins which recognize such sites are the lectins[32]. Lectins encompass a structurally dissimilar class of proteins whose common property is the ability to bind multivalently to specific sugar residues. This ability enables lectins to bind more than one cell, cross-linking them and thus causing them to agglutinate (a process named after the original name given to lectins).

Specific bacteria-host interactions which are mediated by lectins have been demonstrated in at least one case: in the attachment of Rhizobium trifolii to clover root hairs[1]. Infectious strains interact specifically with a lectin produced by the plant, whereas non-infectious strains do not.

Con A, a well-studied lectin, greatly enhances aggregation of a number of oral bacteria, in the presence of saliva[26]. This may be an indication that lectins can promote attachment of cells to plaque and may actually do so in situ[6]. Alternatively, lectins may act to aggregate oral bacteria, thus preventing their attachment to the plaque and promoting their removal from the dental cavity by saliva flow.

In one study, a variety of plant extracts were tested for the ability to agglutinate a number of oral bacteria[3]. While some of the extracts exhibited selective agglutination of bacteria, the extracts were not purified and the molecules responsible for the phenomena were not identified. In a very interesting related study, carrot slices were were found to specifically remove S. mutans cells from a mixed culture, presumably by selectively sorbing the cells to the carrot surface[4]. While lectins may well be involved in the interactions reported above, this has yet to be shown. Two interesting directions are suggested by this possibility:

1. to examine the effect of extracts of edible plants whose lectins are well-characterized and to try to establish a correlation between activity of these lectins and agglutination of cariogenic bacteria.

2. A long list of plants and plant products have been employed by various peoples for thousands of years for chewing purposes. The possibility exists that such masticatory plants may contain compounds which are active in preventing cariogenesis [24]. Chewing sticks, used by the native population of Nigeria, have been shown to possess an antibacterial effect on oral species [10]. Investigations to determine the efficacy of various masticatory plants in limiting the colonization of teeth by oral bacteria would be of great interest. Whether the mechanisms involved are related to inhibition of the specific interactions described in this report can only be postulated.

Competitive Inhibition by Sugar Ligands -

Just as the aggregating activity of antibodies on their specific antigens can be blocked by the addition of haptens, so might the attachment and adherence of bacteria to dental plaque be inhibited by certain sugar molecules which can block the association mechanisms described above.

Fortunately, inhibition by various sugars has been tested in the case of S. mutans. Dextransucrase activity can be inhibited by a variety of sugars, including fructose and maltose [5,16]. The agglutination of S. mutans by dextran is also blocked by fructose, and inhibited by α-methyl glucoside, as well [27]. However, few studies have been undertaken to determine whether sugar ligands can also reverse adherence and agglutination processes in vitro. Such studies would provide an indication of the ability of such molecules to remove cells from plaque after they have become attached.

The prophylactic applicability of specific sugar molecules in preventing dental caries will depend primarily on three factors:

1. High affinity for the recognition sites.
2. Low rate of metabolic degradation.
3. Lack of toxicity.

Thus, although the in vitro studies described above suggest the efficacy of fructose in inhibiting interactions of association and adherence in S. mutans, its relatively high rate of metabolism by oral microorganisms might render it ineffective. On the other hand, experiments with Con A have shown that certain modified mono- and oligo-saccharides can bind to the lectin and block its ability to precipitate glycogen [19,33]. Similarly, experiments with wheat germ agglutinin (WGA), a second lectin, have demonstrated that oligosaccharides of N-acetyl glucosamine of increasing chain lengths has a higher affinity for the lectin than the monosaccharide [32].

The ability of modified monosaccharides and oligosaccharides to specifically bind to lectins and prevent them from agglutinating polysaccharides and cells may prove an important model in future attempts to use modified sugar molecules which are neither toxic nor rapidly metabolized as potential inhibitors of bacterial colonization of teeth. Thus, the prospect of a 'sweet solution' to the problem of dental caries may well exist.

REFERENCES.

1. Bohlool, B.B. and E.L. Schmidt (1974). Science, 185, 269.
2. Bjorn, H. and J. Carlsson (1964). Odontol. Rev. 15, 23.
3. Bratthall,D. (1977). IADR Abstracts 453.
4. Bratthall,D. (1978). In "Secretory Immunity and Infection", (Jerry R. McGhee et al. eds.) Plenum Press, N.Y.
5. Chludzinski, A.M., G.R. Germaine, C.F. Schachtele (1974). J. Bacteriol., 118, 1.
6. Costerton, J.W., G.G. Geesey, K.-J. Cheng (1978). Sci. Am. 238, 86
7. De Stoppelaar,J.D., K.G. Konig, A.J.M. Plasschaert, J.S.van der Hoeven (1971). Arch. Oral Biol. 16, 971.
8. De Stoppelaar, J., J. van Houte, O. Backer-Dirks (1969). Caries Res. 3, 190.
9. Edgar, W.M. (1972). Caries Res. 6, 80.
10. El-Said, F., S.O. Fadulu, J.O. Kuye, and E.A. Sotowora (1971). Lloydia, 34 172.
11. Ericson, T. and J. Magnusson (1976). Caries Res. 10, 8.
12. Freedman, M.L. and Tanzer, J.M. (1974). Infect. Immun. 10, 189
13. Germaine, G.R. and C.F. Schachtele (1976). Infect. Immun. 13, 365
14. Gibbons, R.J., (1977). Microbiology, 395
15. Gibbons, R.J., and J. van Houte (1975). Ann. Rev. Microbiol. 29, 19
16. Gibbons, R.J., and M. Nygaard (1970). Arch. Oral Biol. 15, 1397.
17. Gibbons, R.J., and V. Qureshi (1976), in Microbial Aspects of Dental Caries (W.J. Loesche and H.M. Stiles eds.). Information Retrieval Inc., Washington, D.C.
18. Gibbons, R.J., S.S. Socransky, W.C. de Araujo, and J. van Houte (1964). Arch. Oral Biol., 9, 365.
19. Goldstein, I.J., S. Hammarstrom and A. Misaki (1974). Ann. N.Y. Acad. Sci., 234, 283.
20. Hamada, S. and M. Torii, (1978). Infect. Immun. 20, 592.
21. Hay, D.I., Gibbons, R.J., Spinell, D.M. (1971). Caries Res. 5, 111

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One interesting and important example of this phenomenon is the growth of hydrocarbon utilizing microorganisms on the surfaces of insoluble liquid and solid hydrocarbon (5). Growth of bacteria and yeast on hydrocarbon as a source of carbon and energy has received increasing attention in recent years. Potential applications are sought in such varied fields as food technology (6) and ecology (7). In addition to providing an inexpensive source of single cell protein, hydrocarbon degrading microorganisms and their extracellular products may help provide a biological solution to oil pollution problems.

The adherence of bacteria to hydrocarbon may be viewed as one example of the more general phenomenon of interactions between bacteria and hydrophobic, nonwettable surfaces. Other examples include the adherence of sulfur-oxidizing bacteria to insoluble sulfur particles (8,9) and the attachment of marine bacteria to a variety of hydrophobic surfaces (10-12).

Studies on the growth of microorganisms through direct contact with the bulk hydrocarbon phase are generally complicated by emulsion formation during growth (7). Many hydrocarbon degrading yeast and bacteria release emulsifying agents which "pseudosolubilize" the hydrocarbon phase in the form of microscopic droplets while the culture is being mixed. Reports on the effects of endogenous production or exogenous addition of emulsifying agents on the degree of direct contact between the cells and the bulk hydrocarbon phase are often ambiguous and sometimes contradictory (13-16). A better understanding of the relationship between emulsifier synthesis and direct contact between the cells and the hydrocarbon phase is thus necessary.

In the proposed study, the characteristics and mechanism of adherence of the hydrocarbon-degrading bacterium, RAG-1, to water-insoluble alkanes will be investigated. RAG-1 is able to grow on normal long-chain alkanes and has been observed to adhere to solid hydrocarbon (tetracosane). During growth on a variety of carbon sources, RAG-1 synthesizes and releases into the growth medium a viscous, acidic polysaccharide which is a potent emulsifier (17-21). This macromolecule, termed emulsan, differs from other emulsifying agents which have been reported in hydrocarbon-degrading microorganisms in its relatively high molecular weight, and chemical structure. Further studies on the physical and chemical properties of emulsan are currently under investigation.

In the course of the proposed research, the mechanism by which RAG-1 adheres to hydrophobic surfaces and its possible relationship to emulsan production will be investigated. One hypothesis is that the amphipathic nature of emulsan enables the cell-bound moiety to link the cell to the hydrocarbon surface. A better understanding of the relationship between emulsifying ability and adherence of cells to the hydrocarbon phase will help to clarify the biological role of emulsan.

Attempts will be made to directly observe growth of RAG-1 on an inert hydrophobic surface immersed in nutrient media. It will be of interest to see whether the cells colonize the surface of the substratum without leaving the interface, or become attached and detached during their growth cycle. Attempts will be made to determine whether the main mode of growth of RAG-1 on hydrocarbon in the absence of emulsification occurs through direct contact between the cells and the bulk hydrocarbon phase. Experiments will be carried out to determine whether direct contact between the cells and the hydrophobic surface requires an expenditure of energy or is independent of metabolism.

Reports indicating that various bacteria may adhere to inert, hydrophobic surfaces have been published in recent years (10-12); the biological significance of such interactions remains unclear.

RESEARCH PLAN

An accurate assay has been developed in this laboratory to study the quantitative adherence of bacteria to parrafin based on radioactive labelling and selective filtration techniques (5). Preliminary experiments have led to the modification of an existing assay (12) of bacterial attachment to polystryene, enabling accurate measurement of the extent of bacterial adherence. Initially, optimal conditions for adherence of RAG-1 to both hydrocarbon and inert hydrophobic surfaces will be determined. Conditions will then be sought under which adherence can be prevented or inhibited. Since bacterial adherence is a cell-surface phenomenon, various chemical and enzymatic techniques will be employed to change the composition of the cell surface, and "strip" the cell exterior of various components. The effect of such treatments on adherence will be ascertained.

Mutants of RAG-1, differing from the wild type in emulsan production and various cell-surface characteristics, have been isolated in this laboratory. The ability of each of these mutants to adhere will be determined and correlated to the adherence ability of the wild type. In addition, attempts will be made to enrich for and select "non-sticky" mutants which can no longer adhere to inert hydrophobic surfaces. The ability of such adherence-mutants to grow on hydrocarbon and to produce emulsan will be studied.

The combination of chemical, biochemical and microbiological techniques are expected to provide information on the cell surface components responsible for the adherent abilities of RAG-1. The chemical structure of these components will be investigated with the hope of clarifying the chemical-physical nature of the interactions between RAG-1 and hydrophobic surfaces.

EXPERIMENTAL

Adherence of RAG-1 to hydrocarbon - quantitative assay for cells bound to solid hydrocarbon has been recently developed (5). In this method, the cells are grown in the presence of solid hydrocarbon as the carbon and energy source and radioactive orthophosphate (^{32}Pi) as radioactive label. At given times the growth medium is filtered; bound cells remain on the filter while free cells appear in the filtrate. The distribution of radioactivity in the two fractions is a measure of the ratio of bound to unbound cells.

Adherence of RAG-1 to inert hydrophobic surfaces - bacterial suspensions are placed in contact with the flat, optically clear surface to be studied. At given times the surface is washed extensively and the adherent cells counted microscopically. Surfaces to be investigated include polystryene and Teflon.

Emulsan production - the concentration of emulsan can be routinely determined by a convenient emulsification assay (19).

"Stripping" of the outer cell surface - chemical treatments of cells include ethylene diamine tetraacetic acid, mild alkaline hydrolysis, and incubation of the cells in buffers of extremely low ionic concentrations. Enzymatic treatments include the use of proteolytic enzymes to remove exposed protein moieties, and esterase to hydrolyze fatty-acid esters.

Dependence of adherence on metabolism - the effect of respiratory inhibitors, such as cyanide and azide, and inhibitors of protein synthesis, such as chloramphenicol, on the adherence of RAG-1 will be studied.

Isolation of adherence$^-$ mutants - cells will be grown in the presence of a large surface area of polystryene, under conditions which enable optimal adherence.

Unbound cells will be successively transferred and in this manner the population of non-adherent cells may be enriched.

LOCATION OF RESEARCH

Research will be conducted in the laboratories of the Department of Microbiology, George S. Wise Center for Life Sciences, Tel Aviv University.

REFERENCES

1. Costerton, J.W., G.G. Geesey and K.J. Cheng, 1978. How Bacteria Stick. Scientific American 238, 86.
2. Fletcher, M., Attachment of Marine Bacteria to Surfaces, in Microbiology, D. Schlessinger, ed., 1977. p.407-410. A.S.M. Washington.
3. Gibbons, R.J., 1977. Adherence of Bacteria to Host Tissue: Position Paper, in Microbiology, D. Schlessinger, ed., A.S.M. Washington, p.395-406.
4. Zobell, C.E. 1943. The Effect of Solid Surfaces upon Bacterial Activity. J. Bacteriol. 46, 395.
5. Zilber, I. 1979. Utilization of the Solid Hydrocarbon n-Tetracosane by the Marine Bacterium UP-2. Ph.D. Thesis, Tel Aviv University.
6. Johnson, M.J., 1967. Growth of Microbial Cells on Hydrocarbons. Science, 155, 1515.
7. Gutnick, D.L. and E. Rosenberg, 1977. Oil Tankers and Pollution: A Microbiological Approach. Ann. Rev. Microbiol. 33, 379.
8. Schaeffer, W.I., Holbert, P.E. and Umbreith, W.W., 1963. Attachment of Thiobacillus Thiooxidans to Sulfur Crystals. J. Bacteriol. 85, 137.
9. Schaeffer, W.I., and Umbreit, W.W. 1963. Phosphotidylinosotol as a Wetting Agent in Sulfur Oxidation by Thiobacillus Thiooxidans. J. Bacteriol. 85, 492.
10. Dexter, S.C., J.D. Sullivan, Jr., J. Williams III and S.W. Watson. 1975. Influence of Substrate Wettability on the Attachment of Marine Bacteria to Various Surfaces, Appl. Microbiol. 30, 298.
11. Fletcher, M. and G.I. Loeb, 1979. Influence of Substratum Characteristics on the Attachment of a Marine Pseudomonad to Solid Surfaces, Appl. and Environ. Microbiol. 37, 67.
12. Fletcher, M., 1977. The Effects of Culture Concentration and Age, Time and Temperature on Bacterial Attachment of Polystryene. Can. J. Microbiol. 23, 1.

13. Borzani, W., B.R.V. Conconek, M.L.R. Vairo, P.A. Doin and J. Kovaks, 1971. A Surface Model for the Kinetic Study of Hydrocarbon Utilization by Yeasts in Recent Advances in Microbiology, X. Intl. Congress, Mexico City.

14. Chakravarty, M., P.M. Amin, H.D. Singh, J.N. Baruah and M.S. Iyengar, 1972. A Kinetic Model for Microbial Growth on Solid Hydrocarbons. Biotech. Bioeng. 14, 61.

15. Miura, Y., M. Ozaki, S.I. Hamada, S.I. Murakawa and R. Yugan. 1977. Assimilation of Liquid Hydrocarbons by Microorganisms. I. Mechanisms of Hydrocarbon Uptake. Biotech. Bioeng. 19, 701.

16. Velankar, S.K., S.M. Barnett, C.W. Houston and A.R. Thompson, 1975. Biotech. Bioeng. 17, 241.

17. Horowitz, A., D. Gutnick and E. Rosenberg, 1975. Sequential Growth of Bacteria on Crude Oil. Appl. Microbiol. 30, 10.

18. Reisfeld, A., E. Rosenberg and D. Gutnick, 1972. Microbial Degradation of Crude Oil: Factors Affecting Dispersion in Sea Water by Mixed and Pure Cultures. Appl. Microbiol. 24, 363.

19. Rosenberg, E., A. Zuckerberg, C. Rubinowitz and D.L. Gutnick, 1979. Emulsifier of Arthrobacter RAG-1: Isolation and Emulsifying Properties, Appl. & Environ. Microbiol. 37, 402.

20. Zuckerberg, A., A. Diver, Z. Peeri, D.L. Gutnick and E. Rosenberg, 1979. Emulsifier of Arthrobacter RAG-1: Chemical and Physical Properties. Appl. & Environ. Microbiol. 37, 414.

21. Belsky, I., D.L. Gutnick and E. Rosenberg, 1979. Emulsifier of Arthrobacter RAG-1: Determination of Emulsifier-bound Fatty Acids. FEBS Letters 101, 175.

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Proposal Outline

3/6/79.

Purpose

To determine the mechanism by which RAG-1 can grow through direct contact with the hydrocarbon phase and to study the relationship between direct contact and emulsifier production, using RAG-1 as a model. ~~The following questions will be investigated:~~

Approach

1. Finding a system in which the cells can grow only through direct contact with the H.C. phase.
2. Selecting for a non-sticky mutant which can grow only on emulsified H.C.
3. Developing a simple quantitative system for counting cells bound to the H.C. phase.
4. Determining whether direct contact is mediated by changes ~~on~~ at the cell surface, + attempting to characterize these modifications and their possible relationships to emulsifier production.

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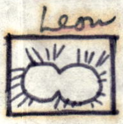
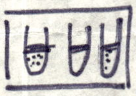
1. Thank Eugene, Dave, dept. members, oil group: Zinaida, Nahum, Yossi, Ed, Ophry, Sarale, Haim, Yuval, Ralph Leon

2. Petroleum microbiology – aside from applied, also basic i.e. (i) how does hc get in? (ii) direct contact vs emulsification.

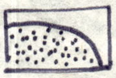
3. RAG-1 as model organism ① identified by Nahum and Ophry as *Acinetobacter calcoaceticus*: ubiquitous organism. advantage (i) sticks during growth on HC (ii) produces emulsifier. Can ask the question – which important?



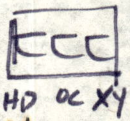
4. Basic adherence assay: ② washed aqueous cell suspension, various volumes of test HC's: HD, OC, XY. vortex two minutes.



③ Cells on droplet surface; can be released by various treatments



Results: ④ RAG-1 vs *E. coli*.

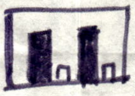


Found: adh not restricted to HC's degrader but related to bacterial hydrophobicity; RAG-1 specificity *P. aeruginosa* not adherent despite HC degrader

5. ⑤ Effect of culture age – important.

6. Adh is not only species specific but also strain specific

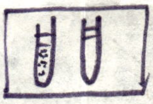
7. Isolation of nonadherent mutant MR-481. Resembles RAG-1 in φ specificity (OPHRY) but doesn't adhere to HC's nor to



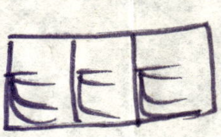
8. ⑥ Epithelial cells – Alex Perry and I. Ofek

9. ⑦ Polystyrene replica.

10. ⑧ Growth on hexadecane RAG-1 vs MR-481.
Method – Klett tubes – Yossi – Low inoculum
add emulsan MR-481 grows.

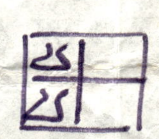


11. MR-481 finally grew? REVERTANTS? Plated, stuck to ps.; two more transfers on HD → RV23-4 ⑨



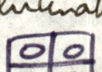
12. Growth of RV1 and RV23-4 on hexadecane.

13. Model for adherence on HC: adh prerequisite for growth under conditions of low initial cell density and absence of emulsification. ⑩



14. ~~Eddie Bayer~~. ~~Story of At~~ Jacob Delarea – no thin pili on MR481.
Eddie Bayer. Mutant AB15. no aggl, no thin pili. ~~[illegible]~~ Missing CIE band
Conditional adherence of AB15. MR-481 – no aggl. RV1, 23-4 much agglutination.

15. ⑪ How ~~ad~~ about Revertant pili?



⑫ CIE

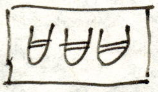
~~⑬ Model for adherence~~ Conc:
① Pili one factor in adh.
② Pili main agglutinogen
③ Role in growth on HC

1. Thank Eugene, Dave, members of the department

2. Petroleum microbiology - aside from applied aspects also basic questions:
i.e. (i.) how does hc get into cell? (ii.) direct contact vs. emulsification.
I solation of petroleum microbiology with respect to other microbiological phenomenon.

3. RAG-1 model organism - sticks, produces emulsifier.
What role sticking? What role of emulsan in sticking?

4. Basic adherence assay: washed cells, no surfactants



Uses: general hydrophobicity
(adh not restricted to hc degraders) - S. marcescens
S. aureus
S. pyogenes
separating cell preparations
isolating mutants

Cells on hydrocarbon droplet

5. Role of culture age in adherence.

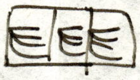
6. Adherence of various Acinetobacter strains: big differences

7. I solation of nonadherent mutant.

10 ~~8.~~ Growth on hexadecane. RAG-1 vs MR481

HOW DONE? FINALLY, GROWTH, CONCLUSIONS

11. ~~9.~~ I solation of partial adherence revertants RV-1, RV23-4

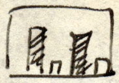


12 ~~10.~~ Renewed growth of RV-1 and RV23-4 on hexadecane

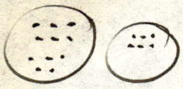
13. ~~11.~~ Model for importance of adherence in growth.

12.

8. Adh to epithelial cells.



9. Adh to polystyrene



14. Similarity between MR-481 and ~~NCIB~~ AB15 — no thin pili
— no aggl.

15. Adh of 481 and 15 on BHI vs EtOH.

16. ~~Model for adherence~~ Pili in RV-1, RV23-4 - regained pili, aggl, partial adh.

17. CIE 18. Model for adherence.

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PROGRESS REPORT: 1981

Adherence of Acinetobacter calcoaceticus RAG-1 to Hydrocarbon and Hydrophobic Surfaces

Submitted by Mel Rosenberg, Department of Microbiology

In the previous report, the development of a quantitative assay for measuring bacterial adherence to hydrocarbon, and the isolation of a non-adherent mutant of RAG-1, named MR-481, were reported. MR-481 was compared with RAG-1 for growth on hexadecane in order to determine whether the loss of adherent ability impairs the ability of the mutant to grow on the insoluble liquid hydrocarbon. In the absence of emulsification and at low initial cell densities, adherent RAG-1 cells grew well on hexadecane, whereas the non-adherent mutant MR-481 was unable to grow under these conditions. These results clearly demonstrated that adherence of bacteria to the insoluble hydrocarbon substrate is essential for growth under conditions similar to those existing in the open environment.

RAG-1 and MR-481 were compared for ultrastructural differences. It was observed under electron microscopic examination that thin filaments which cover the surface of RAG-1 are absent from the surface of the non-adherent mutant. Another mutant existing in the laboratory, termed AB-15, appears to be missing these thin filaments as well. AB-15 was found to be partially deficient in its ability to adhere to hydrocarbons.

Immunological examination of the cell surfaces of wild type RAG-1 and mutants was carried out using agglutination and electrophoretic techniques. While adherent RAG-1 cells were agglutinated by antibodies raised against RAG-1 cells, MR-481 and AB-15 were not agglutinated. These results showed that surface antigens

present on wild type cells were absent from the surfaces of both mutants. In a comparison of RAG-1 and MR-481 using crossed immuno-electrophoresis, at least three distinct antigenic components were revealed to be missing from the surface of MR-481 as compared with RAG-1. These results, taken together with the adherence data, suggest the following model:

(1) The thin filaments protruding from the surface of RAG-1 are responsible for the agglutinability of the wild type cells and some of the adherence properties;

(2) Adherence of RAG-1 to hydrocarbon and to other hydrophobic surfaces is mediated by one or more surface component, acting in addition to or in conjunction with the abovementioned filaments.

(3) The absence of filaments in mutants MR-481 and AB-15 is responsible for their inagglutinability, as well as for partial loss of adherence characteristics.

Further studies are underway in order to test the above model.

In addition, a simple method was devised for screening of hydrophobic colonies on agar plates based on cell adherence to a polystyrene replica. This method distinguishes between colonies of RAG-1 (adherent) and MR-481 (nonadherent) and can be used for the initial screening of bacterial samples for cell surface hydrophobicity and in searching for revertants among nonadherent populations of RAG-1 mutants.

Scientific Publications-

A portion of these data have been published, or are now in press, as follows:

Rosenberg, M., D. Gutnick and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9:29-33.

Rosenberg, M., E. Rosenberg, and D. Gutnick. 1980. Bacterial adherence to hydrocarbons, p. 541-542 In R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R.Rutter, and B. Vincent (ed.) Microbial adhesion to surfaces. Society of Chemical Industry, London.

Rosenberg, M., A. Perry, E.A. Bayer, D.L. Gutnick, E. Rosenberg and I. Ofek. 1981. Adherence of Acinetobacter calcoaceticus RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. 33:29-33.

Rosenberg, M. 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. 42:375-377.

Rosenberg, M., and E. Rosenberg. 1981. Role of adherence in growth of Acinetobacter calcoaceticus RAG-1 on hexadecane. J. Bacteriol. (in press).

Participation in International Meetings.

Two posters summarizing parts of this work were presented at the following meetings:

1) Gordon Conference on Bacterial Cell Surfaces, held in New Hampton, N.H., June 29-July 3, 1981;
2) Fourth International Conference on Surface and Colloid Chemistry, Jerusalem, July 5-10, 1981.

Signature of advisor:

Eugene Rosenberg

Prof. Eugene Rosenberg

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Progress Report: Oct. 1979 - Oct. 1980

Adherence of *Acinetobacter calcoaceticus* RAG-1 to Hydrocarbon and Hydrophobic Surfaces

Submitted by Mel Rosenberg, Dept. of Microbiology

Initial observations indicated that *Acinetobacter calcoaceticus* RAG-1 adhered to the bulk hydrocarbon phase during growth on liquid and solid paraffins. It was of interest to study this phenomenon in terms of the nature of this interaction and the mechanism(s) involved.

A quantitative assay was developed in order to determine bacterial adherence to various liquid hydrocarbons, enabling the following results to be obtained:

1) Various bacteria differed greatly in their ability to adhere to hydrocarbons. No direct correlation was found between the ability of bacteria to degrade hydrocarbon and their ability to adhere to hydrocarbon.

2) In *Serratia marcescens* and *Acinetobacter calcoaceticus* RAG-1, adherence to hydrocarbon was found to increase with culture age.

3) *A. calcoaceticus* RAG-1 was shown to adhere to various hydrocarbons, such as octane and xylene which it is not able to degrade, suggesting that RAG-1 adherence to hydrocarbon may be mediated through a general hydrophobic interaction, rather than specific recognition by the cell of the hydrocarbon moiety.

Experiments which were carried out in collaboration with the laboratory of Dr. Itzhak Ofek (Sackler School of Medicine) have shown that RAG-1 cells adhere avidly to human epithelial cells as well as hydrocarbon, and that a correlation exists between RAG-1 adherence to these two surfaces.

A mutant of RAG-1, deficient in its ability to adhere to hexadecane was isolated. Ultrastructural and chemical comparisons of wild type and mutant cells will hopefully provide data on the mechanism(s) of RAG-1 adherence to hydrophobic surfaces and the cellular structures involved.

Scientific Publications-

A portion of these data have been submitted for publication as follows:

Rosenberg, M., D.L. Gutnick and E. Rosenberg. Bacterial Adherence to Hydrocarbon (accepted for publication in FEMS Microbiological Letters).

Rosenberg, M., E. Rosenberg and D.L. Gutnick. Bacterial Adherence to Hydrocarbons (summary to appear in Symposium on Microbial Adhesion to Surfaces volume).

Rosenberg, M., Perry, A., E.A. Bayer, D.L. Gutnick, E. Rosenberg and I. Ofek. Adherence of *Acinetobacter calcoaceticus* to Epithelial Cells and to a Defined Hydrophobic Surface. (submitted to Infection and Immunity).

Participation in International Meetings-

A poster summarizing a portion of the work described above was presented at the Symposium on Microbial Adhesion to Surfaces, 3-5 Sept., 1980 at Reading, England.

Signatures:

Eugene Rosenberg

Prof. Eugene Rosenberg

Prof. David L. Gutnick

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Effect of Emulsan on Adherence of RAG-1 to Hexadecane

4/6/80

Late log phase RAG-1 cells grown on NB were washed twice and resuspended in PUM to 1.531 OD_{400}. Adherence of cells to 200 μl hexadecane was assayed under the following conditions:

(1) Control – no emulsan added

(2) Emulsan ~~added to cell suspension~~ (AG-12, 100 μg/ml final conc) added to cell suspension prior to mixing.

(3) Emulsan (100 μg/ml) added to test tube following adherence assay; test-tube then mixed for an additional 120 sec.

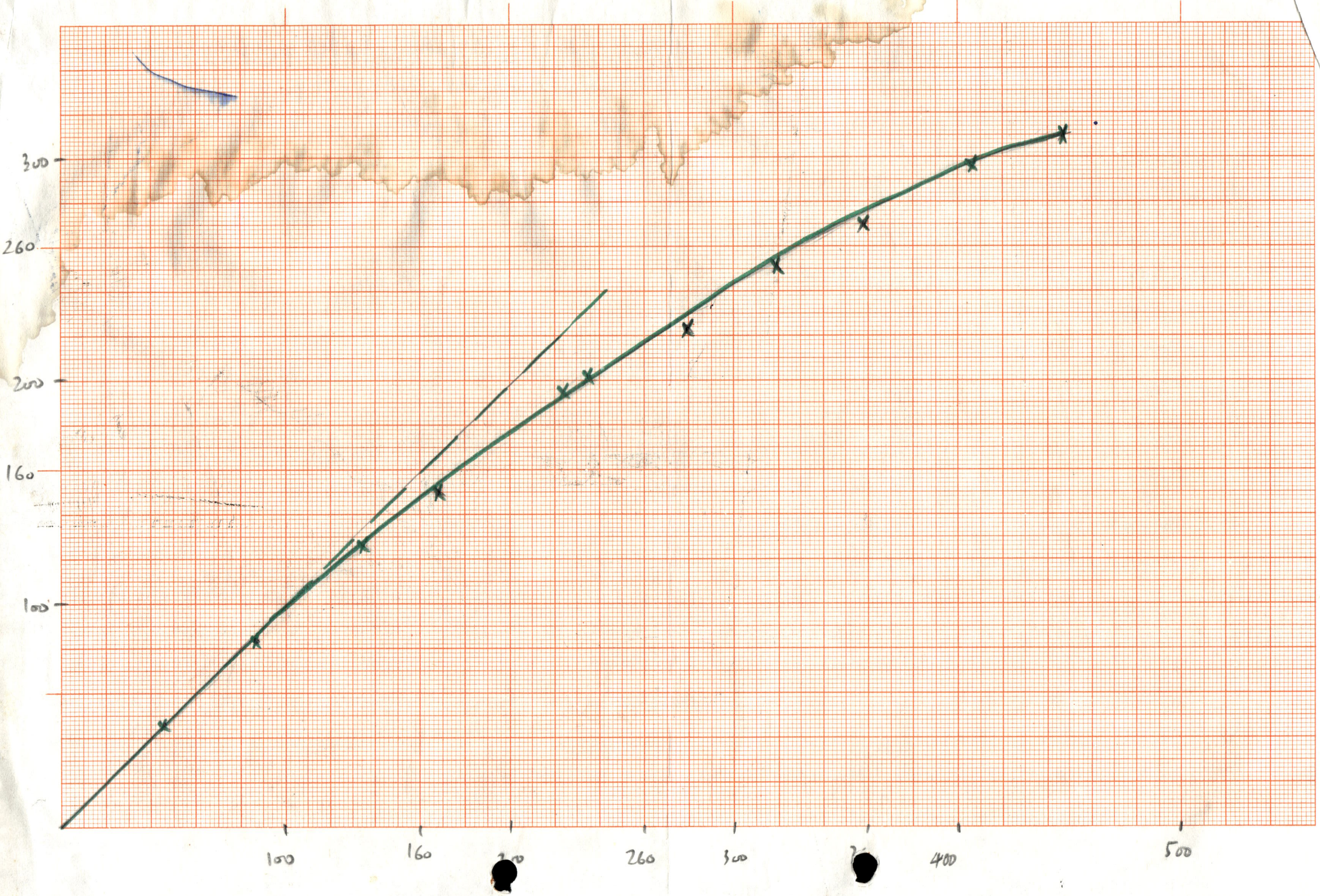
(4) Cells added to test-tube following mixing of emulsan (100 μg/ml final) and hexadecane; test-tube then mixed for an additional 120 sec.

Results –

	O.D.	% turbidity in aqueous phase
Initial	1.531	—
(1)	0.095	6.2
(2)	1.518	104 (corrected)
(3)	1.124	77. "
(4)	1.454	100 "

Conclusions – ① Cells do not adhere to HD droplets stabilized by emulsan. ② Presence of emulsan in assay completoly inhibits adherence of cells. ③ Emulsan removed 82% of cells which had already adhered to HD upon additional mixing.

Corr K.U. 30/12/80



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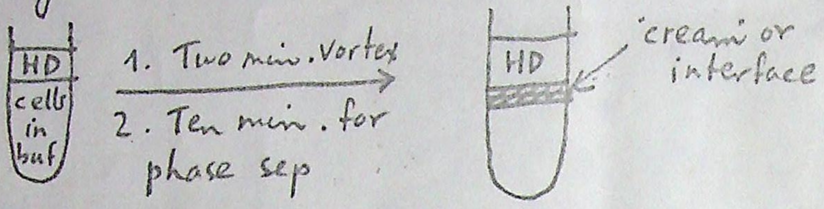
Hexadecane Removal of Cells Suspended in Aqueous Soln.

16/1/80

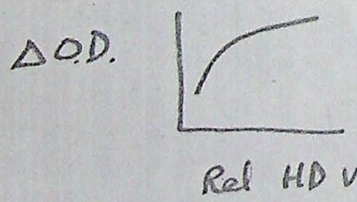
1. Assay:


HD
cells in buf
1. Two min. vortex
2. Ten min. for phase sep
HD
cream or interface

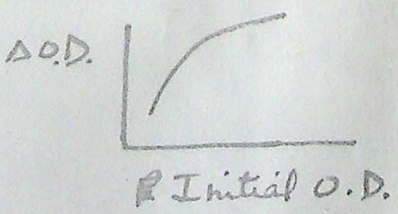
2. Results w/ RAG-1 etc.

	% Removal from aqueous phase	
exptl. cells Rag 1/92	98-99	
old cells - EtOH grown	"	
periodate treated	"	
0.1 M NaOH treated	66 (O.D.) 45 (T.C.)	
in presence of 0.5 ppm Tween 80	~20-30	
312 - Slimy	94	
15φ35	57 O.D. (73 T.C.)	no cream

3. Effect of $\frac{HD}{buffer}$



4. Effect of cell conc.



4. Other Bacteria

	% Removal	Cream
✓ S. aureus	100	large drops
✓ S. albus	0	-
B. cereus	98	large drops
B. subtilis	0	-
P. vulgaris	17	-
E. coli B	1	-
E. coli J5	72	just interface
Gingi	47	just interface

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Progress Report: Oct. 1979 - Oct. 1980

Adherence of *Acinetobacter calcoaceticus* RAG-1 to Hydrocarbon and Hydrophobic Surfaces

Submitted by Mel Rosenberg, Dept. of Microbiology

Initial observations indicated that *Acinetobacter calcoaceticus* RAG-1 adhered to the bulk hydrocarbon phase during growth on liquid and solid paraffins. It was of interest to study this phenomenon in terms of the nature of this interaction and the mechanism(s) involved.

A quantitative assay was developed in order to determine bacterial adherence to various liquid hydrocarbons, enabling the following results to be obtained:

1) Various bacteria differed greatly in their ability to adhere to hydrocarbons. No direct correlation was found between the ability of bacteria to degrade hydrocarbon and their ability to adhere to hydrocarbon.

2) In *Serratia marcescens* and *Acinetobacter calcoaceticus* RAG-1, adherence to hydrocarbon was found to increase with culture age.

3) *A. calcoaceticus* RAG-1 was shown to adhere to various hydrocarbons, such as octane and xylene which it is not able to degrade, suggesting that RAG-1 adherence to hydrocarbon may be mediated through a general hydrophobic interaction, rather than specific recognition by the cell of the hydrocarbon moiety.

Experiments which were carried out in collaboration with the laboratory of Dr. Itzhak Ofek (Sackler School of Medicine) have shown that RAG-1 cells adhere avidly to human epithelial cells as well as hydrocarbon, and that a correlation exists between RAG-1 adherence to these two surfaces.

A mutant of RAG-1, deficient in its ability to adhere to hexadecane was isolated. Ultrastructural and chemical comparisons of wild type and mutant cells will hopefully provide data on the mechanism(s) of RAG-1 adherence to hydrophobic surfaces and the cellular structures involved.

Scientific Publications-

A portion of these data have been submitted for publication as follows:

Rosenberg, M., D.L. Gutnick and E. Rosenberg. Bacterial Adherence to Hydrocarbon (accepted for publication in FEMS Microbiological Letters).

Rosenberg, M., E. Rosenberg and D.L. Gutnick. Bacterial Adherence to Hydrocarbons (summary to appear in Symposium on Microbial Adhesion to Surfaces volume).

Rosenberg, M., Perry, A., E.A. Bayer, D.L. Gutnick, E. Rosenberg and I. Ofek. Adherence of Acinetobacter calcoaceticus to Epithelial Cells and to a Defined Hydrophobic Surface. (submitted to Infection and Immunity).

Participation in International Meetings-

A poster summarizing a portion of the work described above was presented at the Symposium on Microbial Adhesion to Surfaces, 3-5 Sept., 1980 at Reading, England.

Signatures:

Eugene Rosenberg
Prof. Eugene Rosenberg

David L. Gut
Prof. David L. Gutnick

Progress Report: Oct. 1 7[illegible] - Oct. [illegible]

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Initial observations indicate that [illegible]

Rosenberg, M., D. L. Gutnick and E. Rosenberg. Bacterial Adherence to hydrocarbons (accepted for publication in FEMS Microbiological Letters).

[illegible] quantitative assay [illegible]

Rosenberg, M., [illegible] and D. L. Gutnick. Bacterial adherence to hydrocarbons (summary of talk at [illegible] Symposium, in Microbial Adhesion to Surfaces volume).

(1) Various bacteria differed markedly in their ability to adhere to [illegible]

(2) In Serratia marcescens and Acinetobacter calcoaceticus [illegible]

[illegible] calcoaceticus AC-1 was shown to adhere to various hydrocarbons such as octane and xylene which it is not able to degrade, suggesting that AC-1 adherence to hydrocarbon may be mediated through a general hydrophobic interaction, rather than specific recognition by the cell of the hydrocarbon moiety.

Experiments which were carried out in collaboration with the laboratory of Dr. [illegible] (Sackler School of Medicine) have shown that AC-1 [illegible] as well as hydrocarbon and that a correlation exists between AC-1 adherence to these two surfaces.

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ADHERENCE OF *ACINETOBACTER CALCOACETICUS* RAG-1 TO HYDROPHOBIC SURFACES

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Sciences, Tel Aviv University, Ramat Aviv, Israel.

A simple and quantitative method was developed for measuring the adherence of bacteria to liquid hydrocarbons. Although initially developed for the study of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1, the method provides a general technique for measuring cell surface hydrophobicity. Using a modification of the method, a nonadhering mutant referred to as MR-481, was isolated. This mutant was unable to adhere to liquid hydrocarbons, even under conditions which provided optimal adherence in wild-type RAG-1 cells. A second type of mutant, AB-15, was selected for its inability to be agglutinated in the presence of antibodies raised against cell surface components of RAG-1. Strain AB-15 was not adherent to hydrophobic surfaces when grown on minimal medium, but adhered as well as the wild-type when grown on complex medium. Electron microscopic observations revealed that thin filaments, about 30 Å in diameter, which cover the outer surface of stationary phase RAG-1 cells were absent from mutant MR-481 and from mutant AB-15 when grown on minimal medium. The correlation between these filaments and cell-surface hydrophobicity of RAG-1 was further strengthened by experiments using immunoadsorption techniques and crossed-immunoelectrophoresis. Preliminary data indicate that specific surface antigens present on adherent RAG-1 were absent from the mutant cells.

PROPERTIES OF EMULSAN: AN ANIONIC POLYMERIC BACTERIAL EMULSIFIER

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Department of Microbiology, George S. Wise Faculty of Life
Sciences, Tel Aviv University, Ramat Aviv, Israel.

Emulsan is a polymeric (M.W. 9 x 10^5) heteropolysaccharide emulsifying agent purified from the cell-free growth medium of Acinetobacter calcoaceticus RAG-1. The polymer consists largely of N-acetyl D-galactosamine and N-acyl-hexosamine uronic acid (PK 3.05) with an O-ester content of 0.5 μmoles per mg. Studies on the emulsification of hydrocarbons in the presence of emulsan demonstrated: (1) hydrocarbon substrate specificity in which both aliphatic and aromatic or cyclic hydrocarbons are required for optimal emulsions; (2) the formation of microscopic droplets (3-5 microns in diameter) which do not coalesce upon breakage, but rather form stable "creams" which readily disperse in water upon gentle agitation; and (3) an enhancement of binding of cationic dyes to emulsan resulting from the interaction of emulsan with hydrocarbon. Antibodies prepared against purified emulsan were used as specific probes to demonstrate that over 90% of the input emulsan is bound to the hydrocarbon droplets. Antibodies were also used to localize emulsan on the bacterial cell-surface prior to its release into the growth medium. In contrast to the released emulsan, the cell-associated form of the polymer is not active in hydrocarbon emulsification. The specificity of emulsan was also investigated by isolating mutants of RAG-1 defective in extracellular emulsifying activity and comparing the genetically modified extracellular emulsan derivatives with both wild-type emulsan and other emulsion stabilizers.

BACTERIAL ADHERENCE TO HYDROCARBONS - ABSTRACT

March 19th, 1980

A simple, quantitative method has been developed for studying the adherence of microbial cells to immiscible liquid hydrocarbons. In this assay, various volumes of a given liquid hydrocarbon are added to test tubes containing aqueous suspensions of washed cells and the mixtures are subsequently agitated under controlled conditions. Adherent cells bind to the small hydrocarbon droplets and rise with them to form a 'creamy' upper layer. The percentage quantity of adherent cells was ascertained by allowing the phases to separate and then measuring the decrease in light absorption of the aqueous phase. In the present study, various bacteria were grown to early log phase in complex medium, washed, and tested for their ability to adhere to n-hexadecane, n-octane and p-xylene.

A majority of the bacteria tested (*Escherichia coli B* and *K-12*, *Bacillus subtilis*, *Micrococcus lysodeikticus*, *Enterobacter aerogenes*) showed no affinity towards hexadecane or octane and little or no affinity towards xylene. Two hexadecane-degrading strains of *Acinetobacter* exhibited high affinity towards all three hydrocarbons, despite their inability to metabolize octane and xylene. *Staphylococcus aureus*, a microorganism incapable of degrading hydrocarbons, adhered strongly to all three hydrocarbons. These results indicate that (1) the ability to adhere to hydrocarbons is not, as previously suggested, confined to microorganisms capable of hydrocarbon degradation and (2) the adherence of hydrocarbon-degraders to hydrocarbon is not necessarily restricted to those hydrocarbons which they can metabolize.

BACTERIAL ADHERENCE TO HYDROCARBONS

by

M. Rosenberg, E. Rosenberg and D. Gutnick
George S. Wise Faculty of Life Sciences
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A simple quantitative method has been developed for studying the adherence of microbial cells to immiscible liquid hydrocarbons. In this assay, various volumes of a given liquid hydrocarbon are mixed with aqueous suspensions of washed cells under controlled conditions. Adherent cells bind to the small hydrocarbon droplets and separate with them to form an oil in water emulsified upper layer. The adherence of the cells was determined by measuring the decrease in light absorption of the aqueous phase following separation of the phases. In the present study, various bacteria were grown to early log phase in complex medium, washed, and tested for their ability to adhere to n-hexadecane, n-octane and p-xylene. A mojority of the bacteria tested (Escherichia coli B and K-12, Bacillus subtilis, Micrococcus lysodeikticus, Enterobacter aerogenes) showed no affinity towards hexadecane or octane and little or no affinity towards p-xylene. Two hexadecane-degrading strains of Acinetobacter exhibited high affinity towards all three hydrocarbons, despite their inability to metabolize octane and xylene. More than 99% of the cells were found to adhere. Staphylococcus aureus, a microorganism incapable of degrading hydrocarbons, also adhered strongly to all three hydrocarbons. These results indicate that (1) the ability to adhere to hydrocarbons is not, as previously suggested, restricted to microorganisms capable of hydrocarbon degradation and (2) the adherence of hydrocarbon-degrading microorganisms to hydrocarbons is not necessarily restricted to metabolizeable hydrocarbon substrates.

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Project Appraisal - Jan. 2, 1980

I. Thermodynamic Aspects

The outer surface of the bacterial cell confers general physical-chemical properties on the cell, as well as more specific biochemical characteristics. The behaviour of the cell as a particle suspended in liquid is often a function of these general surface properties. The adherence of bacteria to interfaces between aqueous solution and hydrophobic surfaces may be one such example. Such interfaces possess relatively high surface tensions (i.e., 50 dynes/cm for water:octane). It is easily shown that the greater the surface tension between the bacteria and the aqueous phase, the greater the tendency for the cells to partition at the hydrophobic surface. Similarly, if the surface tension at the liquid-hydrophobic surface interface is reduced to values which fall below the surface tension between the cells and the surrounding aqueous phase, free energy considerations no longer favour this adherence. Cells whose outer surface is less hydrophobic in nature (lower surface tension with water) will not tend to adhere to hydrophobic surfaces.

II. Biochemical Aspects

The differences in the surface hydrophobicities of various microbial cells is related to the presence(or absence) of specific chemical structures on the outer surface of the cell. While the concentration and distribution of these structures may depend somewhat on the physiological condition of the cells, drastic changes may not be observable, just as antigenic specificity of bacteria is not greatly influenced by the conditions under which the cells are grown.

III. Relationship to Emulsan

There is now evidence that emulsan which is released into the growth culture exists on the outer cell surface in a bound form prior to its release. This strengthens our original hypothesis that cell-bound emulsan may be primarily involved in enabling the cell to bind and take up hydrocarbon.

IV. Adherence Assays now at our Disposal

1. Quantitative assay for determining adherence of cells to various microscopically-observable surfaces.
2. A quantitative assay for the ability of various non-aqueous liquids to remove cells suspended in the aqueous phase.

V. Related Assays which may be Developed

1. Measurements of contact angles of bacteria and substrata which will establish the relationship between thermodynamic parameters and adherence.
2. Possible use of the ELISA technique to quantitate adherence to substrata which are not microscopically observable.

VI. Experimental Approach:

1. To explore means of changing the outer surface structure of RAG-1 by:
    - a) Chemical methods: EDTA, urea, detergents, NaOH, etc.
    - b) Biochemical means: lipase, trypsin, lysozyme, etc.
    - c) Physical means: i.e., omnimix
    - d) Physiological: Comparison of lipase production, emulsan release,

and adherence during growth.

e) Genetic: selection of mutants deficient in their ability to adhere to hexadecane.

2. Comparison of the adherence of RAG-1 to hydrophobic surfaces with that of other hydrocarbon degraders and non-degraders and existing cell-surface mutants of RAG-1.

3. To examine the role of the substrate in adherence by:
   a) Preparing specific hydrophobic surfaces for adherence assays.
   b) Testing various non-aqueous liquids and mixtures thereof (Is the ability of a particular liquid hydrocarbon or hydrocarbon mixture to remove RAG-1 from the aqueous phase related to their ability to be emulsified by emulsan?)

4. Eventual isolation and characterization of the chemical structure(s) responsible for the hydrophobic nature of the outer cell surface of RAG-1.

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Waxes - Intro

18/5/80

1. What are true waxes: esters of fatty acids and fatty alcohols

2. Distribution in bacteria:

MYCOBACTERIA, NOCARDIA
CORYNEBACTERIUM
MORAXELLA
ACINETOBACTER
SERRATIA MARCESCENS } Perhaps
BACILLUS CEREUS }

3. Location: In ACINETOBACTER, ~~both~~ associated with outer membrane (Thornley et al., Gallagher, Bryn et al.) and in culture medium (Stewart et. al.).

Cell-Free Wax Synthesis in Mycobacterium Tuberculosis:

L. Wang, K. Takayama, D.S. Goldman + H.K. Schnoes

BBA 260, 41-48 (1972).

1. SYSTEM - washed particulate enzyme from glycerol-alanine grown cells

Pi buffer, pH 7.0
$^{14}C_1$-palmityl-SCoA
DTT (dithiothreitol)
NADPH
ATP
Mg^{++}

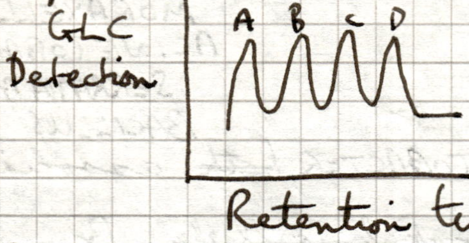
90 min. incubation at 38°C.

2. Identification + Characterization + Purification

1. 30 min. hydrolysis w/ 1M NaOH, room temp (doesn't destroy wax ester).
2. Extraction with DEE.
3. TLC on Silica gel G. Benzene: Chloroform 7:3 V/V
4. GLC on 6% DEXSIL 300 column (310°C), recovery ~~230°-250°C~~ in glass-wool, label retained 5-10 per min. Ester peaks recovered.
5. Extraction with DEE — mass spec
   — Saponified: identification of acid + alcohol + label distribution

Results—

1. In acetone ppttd. ~~is sup~~ prep, 70% of ~~ac~~ label → alcohol.
2. In crude prep major labelled product → wax esters.
dialyzed sup – 25%



Label A – 0
B – 180 cpm
C – 360 cpm
D – ~0

peak B – palmityl palmitate (C_{32})
C – palmityl stearate (C_{34})

LABEL

C_{34} – only alcohol
C_{32} – both (2:1 alcohol:acid)

Conclusions:

1. CoA Fatty acid reduced to alcohol in presence of NADPH + ATP.
Alcohol condenses with endogenous f'as to form wax.
2. In crude membrane prep, major product is wax, but in acetone pptt, major product is alcohol. Something is missing.

Ba.

Questions

1. Mechanism. Possibities: ① direct ② ~CoA ③ plpds.
In Euglena, alcohol was esterified with endogenous ~~A_{14}~~ C_{14}[illegible] only in presence of CoA + ATP — supports ②. In broccoli, evidence for all 3. In M. cerificans, evidence (unpublished for 2 – doesn't say what).
2. Function. Possibility ① storage (as in etiolated Euglena) ② protection (as in plant leaves) ③ adherence.

Introduction

1. What is true wax?
2. Found in bacteria: mycobacteria, ~~nocardia~~, moraxella, acinetobacter, corynebacterium (perhaps Serratia and Bacillus cereus – Kates). In Acinetobacter both cell bound (Thornley) and in medium (Stewart et al.)
Gallagher,
Bryn et al.

3. How formed? Paper.
   1. M. tuberculosis

Membrane prep 105,000 x 90 min

resusp. in 50 mM Pi buffer pH 7.0 + 0.5mM DTT.

Reaction Mixture

1 mg enzyme
100 mM Pi
4 mM ATP
2.5mM NADPH.
1mM $MgCl_2$
0.25 mM DTT.
10 – 30 μM 1-C^{14} palmityl-CoA
in 1 ml., 38°, 90 min.

If acetone powder – 70% alcohol
If particulate – waxesters.
כנראה היה שם אצל
של המבחר "אסטרין".

Wax esters extracted w/ diethyl ether following hydrolysis of reaction mixture in 1M NaOH at room temperature for 30 min. (ester bond intact).

Purification: TLC on Silica gel G with chloroform: methanol 2:1 v/v.

~~Expe – Aich is I.G.~~

GLC: Dexsil300 column, gaschrom Q

fraction trapped in glass wool, eluted with DEE and then ① mass spec.

② Saponification to determine where radioactivity

Identification -

Gallagher:

column chromatography, TLC, GLC.

GLC: wax is cleaved and methyl esters and fatty alcohols identified

1. sodium methylate

Waxes in Bacteria 6/5/80

1) true wax: condensation product of fatty acid and alcohol:

$$ROH + R'OOH \longrightarrow R-O-\overset{\overset{O}{\|}}{R'} + H_2O$$

Alcohol - initial oxidation of alkane - complex hydroxylase linked to one of several electron carrier systems - i.e. cyt. P450 several proteins involved, some particulate

2) when Acinetobacter calcoaceticus is grown on hexadecane, R and R' = 16. i.e. cetyl palmitate.

3) Where does this take place? Although oxidation probably is related to membrane bound electron carriers and requires NADH or NADPH as cofactors, there ~~are~~ were found extracellular build ups of fatty acids, as well as fatty alcohols, so the ~~inactivated~~ non-activated substrates exist in the culture medium.

4) Where is the wax found? Usually in the culture medium. In Nocardia found mostly intracellular. In Acinetobacter found both in culture medium and in OM ~~extract~~...

5) Which bacteria?

	growth on HC.
Corynebacterium	✓
moraxella	
Acinetobacter	✓
S. marcescens ?	} fatty alcohols found.
B. cereus ?	

found C_{34} and C_{32} esters.

In C_{34} (~~st~~ hexadecyl stearate)

only C_{16} labelled (alcohol).

but in C_{32} found mixture of both, mostly alcohol.

Concluded that palmityl CoA is first reduced to alcohol, then alcohol esterified with endogenous + exogenous ~~ac~~ acid to form wax esters.

Direct esterification of fatty ~~acid~~ acyl + alcohol was observed in all three systems (check).

fatty acyl- *could be phospholipid*.

Wax formation inhibited ~~by~~ if ~~dia~~ ~~enzyme prep dialysed or act~~ acetone extracted.

Biochem 9, 1095-(1970) Kolattukudy - euglena.

The product alcohol was esterified with exogenous myristic acid only in the presence of ATP and CoA, showing that esterification requires activated f.a.

Biochem 6 Evidence for "3" mechanisms

1 direct reverse esterification (not physiological)

2 acyl CoA transferase

3 pld or triglyceride transferase

" Activated acid is apparently involved in the synthesis of waxy esters that are excreted into the medium by *M cerificans* when grown on paraffins".

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Project Appraisal - Jan. 2, 1980

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אוניברסיטת תל-אביב
המחלקה למיקרוביולוגיה
====================

הצעה לתכנית מחקר לקראת התואר "דוקטור לפילוסופיה"

הדבקות של החידק Acinetobacter RAG-1 להידרוקרבון ומשטחים הידרופוביים אחרים.

Adherence of Acinetobacter RAG-1 to Hydrocarbon and Other Hydrophobic Surfaces

מוגש ע"י : מלוין רוזנברג

המנחים : פרופ' יוג'ין רוזנברג
פרופ' דוד גוטניק

הננו מאשרים בזה כי אנו מוכנים להנחות את התלמיד מלוין רוזנברג בעבודת המחקר שלו לקראת התואר "דוקטור לפילוסופיה" .

יוג'ין רוזנברג

פרופ' ד. גוטניק   פרופ' י. רוזנברג

ספטמבר 1979

INTRODUCTION

The adherence of bacteria to a wide variety of inorganic surfaces has been well established, both in natural environments and in the laboratory (1-4). The advantage gained by "sticky" bacteria is perhaps most evident under circumstances in which the cells are able to adhere to surfaces which can provide necessary nutritional sources for metabolism and growth.

One interesting and important example of this phenomenon is the growth of hydrocarbon utilizing microorganisms on the surfaces of insoluble liquid and solid hydrocarbon (5). Growth of bacteria and yeast on hydrocarbon as a source of carbon and energy has received increasing attention in recent years. Potential applications are sought in such varied fields as food technology (6) and ecology (7). In addition to providing an inexpensive source of single cell protein, hydrocarbon degrading microorganisms and their extracellular products may help provide a biological solution to oil pollution problems.

The adherence of bacteria to hydrocarbon may be viewed as one example of the more general phenomenon of interactions between bacteria and hydrophobic, nonwettable surfaces. Other examples include the adherence of sulfur-oxidizing bacteria to insoluble sulfur particles (8,9) and the attachment of marine bacteria to a variety of hydrophobic surfaces (10-12).

Studies on the growth of microorganisms through direct contact with the bulk hydrocarbon phase are generally complicated by emulsion formation during growth (7). Many hydrocarbon degrading yeast and bacteria release emulsifying agents which "pseudosolubilize" the hydrocarbon phase in the form of microscopic droplets while the culture is being mixed. Reports on the effects of endogenous production or exogenous addition of emulsifying agents on the degree of direct contact between the cells and the bulk hydrocarbon phase are often ambiguous and sometimes contradictory (13-16). A better understanding of the relationship between emulsifier synthesis and direct contact between the cells and the hydrocarbon phase is thus necessary.

In the proposed study, the characteristics and mechanism of adherence of the hydrocarbon-degrading bacterium, RAG-1, to water-insoluble alkanes will be investigated. RAG-1 is able to grow on normal long-chain alkanes and has been observed to adhere to solid hydrocarbon (tetracosane). During growth on a variety of carbon sources, RAG-1 synthesizes and releases into the growth medium a viscous, acidic polysaccharide which is a potent emulsifier (17-21). This macromolecule, termed emulsan, differs from other emulsifying agents which have been reported in hydrocarbon-degrading microorganisms in its relatively high molecular weight, and chemical structure. Further studies on the physical and chemical properties of emulsan are currently under investigation.

In the course of the proposed research, the mechanism by which RAG-1 adheres to hydrophobic surfaces and its possible relationship to emulsan production will be investigated. One hypothesis is that the amphipathic nature of emulsan enables the cell-bound moiety to link the cell to the hydrocarbon surface. A better understanding of the relationship between emulsifying ability and adherence of cells to the hydrocarbon phase will help to clarify the biological role of emulsan.

Attempts will be made to directly observe growth of RAG-1 on an inert hydrophobic surface immersed in nutrient media. It will be of interest to see whether the cells colonize the surface of the substratum without leaving the interface, or become attached and detached during their growth cycle. Attempts will be made to determine whether the main mode of growth of RAG-1 on hydrocarbon in the absence of emulsification occurs through direct contact between the cells and the bulk hydrocarbon phase. Experiments will be carried out to determine whether direct contact between the cells and the hydrophobic surface requires an expenditure of energy or is independent of metabolism.

Reports indicating that various bacteria may adhere to inert, hydrophobic surfaces have been published in recent years (10-12); the biological significance of such interactions remains unclear.

RESEARCH PLAN

An accurate assay has been developed in this laboratory to study the quantitative adherence of bacteria to parrafin based on radioactive labelling and selective filtration techniques (5). Preliminary experiments have led to the modification of an existing assay (12) of bacterial attachment to polystryene, enabling accurate measurement of the extent of bacterial adherence. Initially, optimal conditions for adherence of RAG-1 to both hydrocarbon and inert hydrophobic surfaces will be determined. Conditions will then be sought under which adherence can be prevented or inhibited. Since bacterial adherence is a cell-surface phenomenon, various chemical and enzymatic techniques will be employed to change the composition of the cell surface, and "strip" the cell exterior of various components. The effect of such treatments on adherence will be ascertained.

Mutants of RAG-1, differing from the wild type in emulsan production and various cell-surface characteristics, have been isolated in this laboratory. The ability of each of these mutants to adhere will be determined and correlated to the adherence ability of the wild type. In addition, attempts will be made to enrich for and select "non-sticky" mutants which can no longer adhere to inert hydrophobic surfaces. The ability of such adherence-mutants to grow on hydrocarbon and to produce emulsan will be studied.

The combination of chemical, biochemical and microbiological techniques are expected to provide information on the cell surface components responsible for the adherent abilities of RAG-1. The chemical structure of these components will be investigated with the hope of clarifying the chemical-physical nature of the interactions between RAG-1 and hydrophobic surfaces.

EXPERIMENTAL

Adherence of RAG-1 to hydrocarbon - quantitative assay for cells bound to solid hydrocarbon has been recently developed (5). In this method, the cells are grown in the presence of solid hydrocarbon as the carbon and energy source and radioactive orthophosphate (^{32}Pi) as radioactive label. At given times the growth medium is filtered; bound cells remain on the filter while free cells appear in the filtrate. The distribution of radioactivity in the two fractions is a measure of the ratio of bound to unbound cells.

Adherence of RAG-1 to inert hydrophobic surfaces - bacterial suspensions are placed in contact with the flat, optically clear surface to be studied. At given times the surface is washed extensively and the adherent cells counted microscopically. Surfaces to be investigated include polystryene and Teflon.

Emulsan production - the concentration of emulsan can be routinely determined by a convenient emulsification assay (19).

"Stripping" of the outer cell surface - chemical treatments of cells include ethylene diamine tetraacetic acid, mild alkaline hydrolysis, and incubation of the cells in buffers of extremely low ionic concentrations. Enzymatic treatments include the use of proteolytic enzymes to remove exposed protein moieties, and esterase to hydrolyze fatty-acid esters.

Dependence of adherence on metabolism - the effect of respiratory inhibitors, such as cyanide and azide, and inhibitors of protein synthesis, such as chloramphenicol, on the adherence of RAG-1 will be studied.

Isolation of adherence$^-$ mutants - cells will be grown in the presence of a large surface area of polystryene, under conditions which enable optimal adherence.

Unbound cells will be successively transferred and in this manner the population of non-adherent cells may be enriched.

LOCATION OF RESEARCH

Research will be conducted in the laboratories of the Department of Microbiology, George S. Wise Center for Life Sciences, Tel Aviv University.

REFERENCES

1. Costerton, J.W., G.G. Geesey and K.J. Cheng, 1978. How Bacteria Stick. Scientific American 238, 86.
2. Fletcher, M., Attachment of Marine Bacteria to Surfaces, in Microbiology, D. Schlessinger, ed., 1977. p.407-410. A.S.M. Washington.
3. Gibbons, R.J., 1977. Adherence of Bacteria to Host Tissue: Position Paper, in Microbiology, D. Schlessinger, ed., A.S.M. Washington, p.395-406.
4. Zobell, C.E. 1943. The Effect of Solid Surfaces upon Bacterial Activity. J. Bacteriol. 46, 395.
5. Zilber, I. 1979. Utilization of the Solid Hydrocarbon n-Tetracosane by the Marine Bacterium UP-2. Ph.D. Thesis, Tel Aviv University.
6. Johnson, M.J., 1967. Growth of Microbial Cells on Hydrocarbons. Science, 155, 1515.
7. Gutnick, D.L. and E. Rosenberg, 1977. Oil Tankers and Pollution: A Microbiological Approach. Ann. Rev. Microbiol. 33, 379.
8. Schaeffer, W.I., Holbert, P.E. and Umbreith, W.W., 1963. Attachment of Thiobacillus Thiooxidans to Sulfur Crystals. J. Bacteriol. 85, 137.
9. Schaeffer, W.I., and Umbreit, W.W. 1963. Phosphotidylinosotol as a Wetting Agent in Sulfur Oxidation by Thiobacillus Thiooxidans. J. Bacteriol. 85, 492.
10. Dexter, S.C., J.D. Sullivan, Jr., J. Williams III and S.W. Watson. 1975. Influence of Substrate Wettability on the Attachment of Marine Bacteria to Various Surfaces, Appl. Microbiol. 30, 298.
11. Fletcher, M. and G.I. Loeb, 1979. Influence of Substratum Characteristics on the Attachment of a Marine Pseudomonad to Solid Surfaces, Appl. and Environ. Microbiol. 37, 67.
12. Fletcher, M., 1977. The Effects of Culture Concentration and Age, Time and Temperature on Bacterial Attachment of Polystryene. Can. J. Microbiol. 23, 1.

13. Borzani, W., B.R.V. Conconek, M.L.R. Vairo, P.A. Doin and J. Kovaks, 1971. A Surface Model for the Kinetic Study of Hydrocarbon Utilization by Yeasts in Recent Advances in Microbiology, X. Intl. Congress, Mexico City.

14. Chakravarty, M., P.M. Amin, H.D. Singh, J.N. Baruah and M.S. Iyengar, 1972. A Kinetic Model for Microbial Growth on Solid Hydrocarbons. Biotech. Bioeng. 14, 61.

15. Miura, Y., M. Ozaki, S.I. Hamada, S.I. Murakawa and R. Yugan. 1977. Assimilation of Liquid Hydrocarbons by Microorganisms. I, Mechanisms of Hydrocarbon Uptake. Biotech. Bioeng. 19, 701.

16. Velankar, S.K., S.M. Barnett, C.W. Houston and A.R. Thompson, 1975. Biotech. Bioeng. 17, 241.

17. Horowitz, A., D. Gutnick and E. Rosenberg, 1975. Sequential Growth of Bacteria on Crude Oil. Appl. Microbiol. 30, 10.

18. Reisfeld, A., E. Rosenberg and D. Gutnick, 1972. Microbial Degradation of Crude Oil: Factors Affecting Dispersion in Sea Water by Mixed and Pure Cultures. Appl. Microbiol. 24, 363.

19. Rosenberg, E., A. Zuckerberg, C. Rubinowitz and D.L. Gutnick, 1979. Emulsifier of Arthrobacter RAG-1: Isolation and Emulsifying Properties, Appl. & Environ. Microbiol. 37, 402.

20. Zuckerberg, A., A. Diver, Z. Peeri, D.L. Gutnick and E. Rosenberg, 1979. Emulsifier of Arthrobacter RAG-1: Chemical and Physical Properties. Appl. & Environ. Microbiol. 37, 414.

21. Belsky, I., D.L. Gutnick and E. Rosenberg, 1979. Emulsifier of Arthrobacter RAG-1: Determination of Emulsifier-bound Fatty Acids. FEBS Letters 101, 175.

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Research Proposal - First draft 26/5/79

Introduction -

While it has often been customary to study the
behaviour of microorganisms in liquid culture,
in the past decade much attention has been directed
towards ~~the~~ interactions between bacteria and
non-aqueous phases. In liquid culture, the
predominant interactions are between the cells
and the aqueous phase. In nature, however,
may
cells interact with a variety of phases, including
immiscible in water
air, solids, and insoluble liquids. Moreover,
the ability of microorganisms to interact at
vaious
certain interfaces and to adhere to surfaces
presents them with clear ecological advantages,
and is, in some cases, necessary for their
survival. and proliferation.

flora
Bacteria in the oral cavity adhere to the tooth
surface and to epithelial cells. Microorganisms
in flowing rivers and streams are ableto ~~xx~~ hold
fast to rocks and solid surfaces. Rhizobium sp.
adhere to the root hairs of leguminous plants.
Direct contact between bacteria which depend on
sulfur as a source of energy and insoluble sulfur
particles is necessary for growth.

sticking & ecological niche

upon
Bacteria which depend on insoluble substrates face the following ~~p~~ unique problem: in the absence of mechanisms ~~such as~~ bulk transport mechanisms
insoluble
such as phagocytosis, the substrate must be transferred into the cell where it can then be metabolized.

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28/5/78.

Bacteria

Microorganisms which depend upon water-insoluble substrates for growth have a unique problem. They must gain access to such materials and bring them into the cell without resorting to bulk transport tactics, such as phagocytosis.

One such system involves growth of Thiobacilli in the presence of insoluble sulfur particles as sole source of sulfur and energy. In such a system it was observed that direct contact between bacteria and sulfur is necessary for growth (Vogler, K.G., Umbreit, W.W., 1941, Soil Sci. 51, 331-7), and bacteria can be seen clinging tothe surface of crystals of sulfur Waksman, S.S., 1932. Principles of Soil Microbiology. Baltimore: Williams and Wilkins, 2nd ed.), (Schaeffer, W.I., Holbert, P.E, Umbreit, W.W. 1963. J.Bacteriol. 85, 137)

~~Another~~

Growth of microorganisms on insoluble hydrocarbons is another such system, and one with considerable technological and ecological implications (Gutnick & Rosenberg, Ann Rev. Microbiol. 1977, 31 , 379).

The manner in which hydrocarbons are assimilated by microorganisms has been studied to a great extent in yeast, and to a lesser extent in bacteria. In many such systems, direct contact between the cells and the oil phase can be observed during growth. In some systems, direct contact is necessary for growth, and under conditions in which the surface area of the oil-water interface is lowered, the rate of growth also decreases. On the other hand, studies of batch cultures of Candida lipolytica, showed that about 30% of the cells were not bound to large oil droplets, and that the growth rate of adsorbed and detached cells was identical. Similar results have been obtained by Zilber who stduied the growth of a marine pseudomonad, UP2, on solid paraffin (n-tetracosane, m.p.). A model which proposes that a dynamic relationship exists between adsorbed and desorbed cells has been proposed to account for the identical growth rate.

Another mechanism which enables microorganisms to grow on hydrocarbons is by pseudosolubilization or"accomodation" of the insoluble phase, or, simply, emulsification. A wide variety of petroleum-degrading microorganisms have been found to bring about the formation of oil in water emulsions while growing on hydrocarbons (Haldom, R.S. Turner, A.G. 1969, J. Apl. Bacteriol. 32, 448)Rag72 Zajic, J.E., Panchal, C.J. 1976 Crit Rev. Rmicobiol. 5, 39) Zobell, C.E., Ahearn,etc. (G&R 3).

P.K. Roy, HD Singh, S.D. Bhagat + J.N. Baruah 1979 Biotech Bioeng XXI, 955

A number of microorganisms have been found to excrete emulsifying factors into the medium while growing on hydrocarbons.

The models of hydrocarbon assimilation by direct contact and pseudosol emulsification are far from being mutually exclusive. Emulsification of the oil phase considerably reduces the size of the stabilized droplets, thus increasing the surface area available for direct contact. Moreover, direct contact may depend on a reduction in the interfacial tension between the oil and water phase, which is characteristic of emulsifi~~ers~~ying agent.

Proposal

~~It is the purpose of the planned research~~

RAG 1 is a gram negative bacterium which was isolated by virtue of its growth on crude oil. RAG 1 can grow on a variety of paraffins, ~~ranging from to~~ . This bacterium produces an extracellular polymer with potent emulsifying properties ~~which is currently being~~ whose molecular composition is currently under investigation.

The intended purpose of this research project is to study the adherence of RAG-1 cells to the hydrocarbon phase during growth, and the relationship between this adherence and the production of extracellular emulsifier. Answers will be sought to the following questions:

1. Does the ability of the cells to adhere to the hydrocarbon phase depend upon the conditions under which the cells are grown?

2. What, if any, is the relationship between the ability of the cells to synthesize and release emulsifier into the medium, and the tendency of the cells to adhere to the parrafin phase?

3. What is the ratio of bound to unbound cells under various physiological conditions?
4. Does a dynamic relationship exist between bound and unbound cells during growth?

5. Can a class of mutants be isolated which are unable to utilize hydrocarbons because they are unable to adhere to the hydrocarbon phase?

The intended purpose of this research project is to study the adherence of RAG-1 cells to the hydrocarbon phase during growth, and the relationship between this adherence and the production of extracellular emulsifier. Answers will be sought to the following questions:

1. Does the ability of the cells to adhere to the hydrocarbon phase depend upon the conditions under which the cells are grown?

2. What, if any, is the relationship between the ability of the cells to synthesize and release emulsifier into the medium, and the tendency of the cells to adhere to the paraffin phase?

3. What is the ratio of bound to unbound cells under various physiological conditions?

4. Does a dynamic relationship exist between bound and unbound cells during growth?

5. Can a class of mutants be isolated which are unable to utilize hydrocarbon because they are unable to adhere to the hydrocarbon phase?

It is the purpose of the planned research to study

the ~~relationship between th~~e adherence of RAG-1 cells

and relationship with

to hydrocarbons during gro~~x~~wth ~~and it~~s production of

the extracellular emulsifier. The project will be

aimed at answering the following questions?

Is the adhere

Does the adherence of the cells

Does the ability of the cells to adhere to the hydrocarbon phase depend on the conditions under which the cells are grown?

Does the ability of the cells to synthesize and release emulsifier into the medium correlate in some manner the ability of the cells to adhere to the paraffin phase?

~~Is the adherence of the cel~~ls

What is the proportion of

What is the ratio of bound to unbound cells under conditions in which emulsification cannot take place?

Does a dynamic relationship exist between adhering and detached cells.

Can a class of mutants be isolated which are ~~xxx~~ unable to utilize hydrocarbons because they are unable to adhere to the hydrocarbon phase?

The following methods and techniques will be employed during the course of the proposed research:

A. Methods for Separation and Quantitization of cells bound to the hydrocarbon phase - I. Zilber has developed a quantitative method for separation and enumerating cells bound to the hydrocarbon phase as well as separation the cells in the aqueous phase. ~~This technique is based upon filtration~~ The cells are grown in scintillation vials, each vial serving as a single assay. Growth is measured by ^{32}Pi incorporation into TCA insoluble material. The bound cells can be separated from the cells in the aqueous phase by filtration ~~and are then returned to the vial for counting~~. From the distribution of ^{32}Pi incorporation into bound and free cells viable counts of the cells in the aqueous phase, the number of cells bound to the unfilterable parrafin can be determined.

In addition, a method will be developed using membrane filters as an inert support for the hydrocarbon phase. The number of cells which bind to the filter can be counted directly. This method can be used as an accurate and rapid assay for determining the ratio of bound to unbound cells in cultures of low cell concentration.

B. Concentration of Dissolved Hydrocarbon in the aqueous phase will be determined by GLC.

C. Selection of Non-adherent Mutants-

The selection of mutants which cannot grow on paraffins ~~due to~~ because they lack the ability to maintain direct contact with the hydrocarbon phase will depend on a number of premises:

A number of approaches are available.

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Research Proposal June 3rd/79

Introduction

Microbial utilization of petroleum and petroleum
by-products as substrates for growth has ~~xxxxxxxyx~~
attention
received increasing interest in recent years. This
is due primarily to ~~its~~ potential applications in
the fields of ecology and food technology. Ilana 1-7 (5-7 8 9 11)
Microorganisms which metabolize hydrocarbons may
provide an inexpensive source of single cell protein; Ilana 1-3
in addition, such microorganisms and their extracellular
products may provide a biological solution to oil
pollution problems. (6,7)

In order to metabolize substrate, the microbial
cell must be able to gain access to the compound,
transport presents
and bring it into the cell. This becomes a problem
when the substrate is completely, or nearly completely
most
insoluble in water; ~~axxt~~ aliphatic compounds
fall into this category. Thus, in addition to
the water and air phases with which growing micro-
organisms generally contend, hydrocarbon-metabolizing
microorganisms must contend with ~~txix~~ an additional,
immiscible, hydrocarbon phase as well.

Thus, growth of microorganisms on insoluble paraffins presents problems, both for the microorganism and for the researchers studying the phenomenon.

Ilana PhD The manner in which paraffins are assimilated
Ilana 64 by microorganisms has been studied under various
64A conditions in both yeast and bacteria. In many
58 such systems, direct contact between the cells and
41 the oil phase may be observed microscopically
cases
56 during growth. In some systems, 52
direct contact appears to be necessary for growth,
52 some
31 In many studies, microorganisms can be
15 observed to grow both attached to the oil phase,
54 and free or in clumps in the aqueous phase.
52 Moreover, studies of batch cultures of Candida lipolytica, showed that about 30% of the growing cells were not bound to large oil ~~drp~~ droplets, and that the growth rate of adsorbed and detached cells was the same. 58 Similar results were obtained by Zilber for the gorwth of a marine
alkane
pseudomonad, UP-2, on solid paraffin. Ilana PhD, ~~These~~ Such observations led these researchers to suggest that binding to the oil droplets is reversible

and that a dynamic relationship exists between adsorbed and desorbed cells.

Appendages

Microorganisms growing on hydrocarbons possess extracellular structures; ~~to~~ their direct role in adherence, however, has yet to be demonstrated. Ilana! 21, 97, 98 Ilana PhD Ed Bayer. + Fletcher + Floodgate.

Many Microorganisms which degrade hydrocarbons have been found to secrete ~~a variety of~~ substances which bring about oil-in-water emulsions. In such emulsions, the oil becomes "pseudosolubilized", or "accomodated" in the aqueous phase, in the form of microscopic droplets. It has been postulated that such emulsifiers 12/6. as surfactants act to stabilize microscopic droplets in the ~~q~~ aqueous phase and thus provide a greater oil surface area on which the microorganisms can grow. ~~Addxtxxnxxf~~ While the addition of surface active agents can bring about increased growth rates, in some cases they can also cause desorption of cells from the hydrocarbon droplets to which they were previously attached. (Ilana 64).

RAG - 1 is a gram negative, aerobic, non-motile bacterium which was isolated as a degrader of crude oil from sea water. During growth of Rag-1 on solid + liquid hydrocarbons, cells are observed to grow on the hydrocarbon surface, as well as free + in clumps in the aqueous phase. RAG-1 produces an extracellular emulsifying agent when grown on hexadecane, ~~xh~~ ethanol or acetate. This ~~emulsifying~~ expolysaccharide agent, known as emulsaan, is an acidic polysaccharide with ~~has~~ unique and interesting properties; it can be purified by Am Sulf pptn + Phenol ext. its chemical ~~composition~~ structure is now under investigation in this laboratory.

While the role of emulsan in enhancing the growth of RAG-1 on hydrocarbon ~~by~~ through emulsification has been suggested, the apparently gratuitous secretion of large amounts of the biopolymer during growth of RAG-1 on soluble substrates such as ethanol has raised some questions in this regard.

In the proposed research project, the relationship between the ability of RAG-1 cells to grow directly on hydrocarbon and its production of emulsifier will be investigated. The role that emulsan does (or does not) play in the adherence of growing RAG-1 cells to the hydrocarbon phase may shed some light on the ongoing controversy concerning the manner in which insoluble hydrocarbons are assimilated by microorganisms.

... the mechanism by which RAG-1 cells adhere and grow directly on the hydrocarbon phase will be studied. The role which emulsan does (or does not) play in this adherence phenomenon will be investigated. Hopefully, the proposed research.

Purpose of the Proposed Research-

1. To ~~determine~~ study the mechanisms by which microorganisms are able to adhere to the hydrocarbon surface during growth, using RAG-1 as a model; and

2. To investigate the ~~xxxx~~ possible role of emulsan in this process, with the hope of elucidating possible biological functions of the emulsifier.

Approach-

1. Investigation of emulsan production under various growth conditions: Generally, studies which have been carried out have been aimed at maximalizing emulsan production. In this study, growth conditions ~~will be sought~~ under which active emulsan is not produced will be sought. The~~se studies will~~ be carried ~~out in two directions~~:

   a. source of carbon and energy - although high emulsan yields are produced on acetate and ethanol, cells grown growth on Kreb cycle intermediates or amino acids may not synthesize active emulsifier. exopolysaccharide formation is favoured by when excess ~~carbohydrate~~ C source is present + certain other nutrients are limiting (p57 - Duguid '1X).

   b. Limiting Mg^{++} concentration - emuls~~ifier~~an

   Mg^{++} is important factor in integrity of Gram- cell envelope and is bound by teichoic acids and anionic ps

in ethanol + acetate grown cells glyoxylate cycle is present +

Go into more detail here.

Purposes of the Proposed Research-

1. To investigate growth of RAG-1 on hydrocarbon

more detail

production and activity are both dependent on divalent cation. Low concentrations of Mg^{++} in the culture medium may suffice for growth, yet inhibit emulsan synthesis.

C. Limiting carbon substrate
D. staying in log phase

polyamine - Sullivan p. 743

~~2. Finding normal alkanes which do not support growth of~~

ref: Galanos + Lüderitz 1975.

~~2. Use of Mutants-~~

2. Methods for Quantitative Measurement of Bacterial Adherence to Hydrocarbons- A number of methods exist for determining the degree of ~~the~~ adherence of microorganisms for the hydrocarbon phase. These include centrifugation of hydrocarbon-bound cells, various filtration methods, and extraction of bound hydrocarbon. Zilber has developed an elegant quantitative method based on filtration of cells grown on solid hydrocarbon ~~and the enumeration~~ followed by quantitative estimation of the fraction of bound cells by ^{32}Pi incorporation into TCA insoluble fraction. The latter method enables accurate measurement of the fraction of tetracosane-bound cells, but the free cells which pass through the filter still ~~contain~~ carry with them significant amounts of hydrocarbon. ~~In addition~~, In addition, during growth on tetracosane, emulsification takes place and its affect on the degree of adherence is difficult to ascertain.

production and activity are both dependent on divalent cation. Low concentrations of Mg^{++} in the culture medium may suffice for growth, yet inhibit emulsan synthesis.

2. Finding normal alkanes which do not support growth of

3. Use of [illegible]-

. Methods for quantitative measurement of bacterial adherence to hydrocarbons- A number of methods exist for determining the degree of ~~xxx~~ adherence of microorganisms for the hydrocarbon phase. These include centrifugation of hydrocarbon-bound cells, various filtration methods, and extraction of bound hydrocarbon. [illegible] has developed an elegant quantitative method based on filtration of cells grown on solid hydrocarbon ~~and the enumeration~~ followed by quantitative estimation of the fraction of bound cells by [illegible] incorporation into TCA-insoluble fraction. The latter method enables accurate measurement of fraction of the tetracosane-bound cells, but the free cells which pass through the filter still ~~contain~~ carry with them significant amounts of hydrocarbon. In addition, during growth on tetracosane, emulsification takes place and its effect on the degree of adherence is difficult to ascertain.

A method will be developed to quantitatively ~~determ~~ enumerate bacteria which adhere to hydrocarbon under
which prevent emulsigification of the hc
conditions in which emulsification of the hydrocarbon ~~phase is negligible~~ moiety . Such a system is plausible if the hydrocarbon is adsorbed to an inert support, such as filters. Such filters, impregnated with small amounts of hydrocarbon, will be floated on aqueous medium. In
will be
such a system, cells ~~are~~ able to grow on the hydrocarbon only when directly in contact with the filter. At various times, filters may be removed from the medium,. clarified, and the adherent cells enumerated visually. The successful development of such a system will enable the following questions to be attacked experimentally?

1. What is the ratio of bound to unbound cells during growth when the hydrocarbon on the filter is the only source of carbon and energy?
2. What is the ratio of bound to unbound cells ~~during~~ Measurement of the ratio of bound to unbound cells and the growth rates as a function of:
    - a) the hydrocarbon substrate
    - b) salt (particularly Mg^{++}) concentration
    - c) limiting concentrations of soluble substrates
in the presence of
(i.e., can a two-phase growth be observed when limiting concentrations of soluble concentrations is there a lag under such conditions

3. ~~d)~~ what is the relationship between adherence and metabolism? Will the cells adhere to

hydrocarbon which they are incapable of metabolizing? ~~Will the cells a~~ Will adherent cells be adsorbed by addition of metabolic inhibitors? Will the initial adherence of cells be prevented by metabolic inhibitors?

4. Do cells adhere to the hydrocarbon phase under conditions in which no emulsification activity is observable.

(What is the effect) ....

5. Can the addition of external emulsifiers (both emulsan and synthetic surfactants) prevent adherence or desorb adherent cells?

6. Mutants have been isolated in this laboratory which produce ~~[illegible]~~ very low levels of emulsifying activity ~~emulsan~~ or altered exopolysaccharide. It will be interesting to see whether these mutants ~~are~~ exhibit changes in growth and adherence to hydrocarbon under the experimental conditions.

7. Non-sticky mutants (see section) should exhibit reduced adherence and growth under the experimental conditions. It will be interesting to see whether addition of emulsan and/or surfactants can reverse the altered phenotype.

8. Will addition of low concentrations of Various ~~certain~~ lytic enzymes (proteases, lipases, esterases) inhibit prevent adherence and growth underthe experimental conditions?

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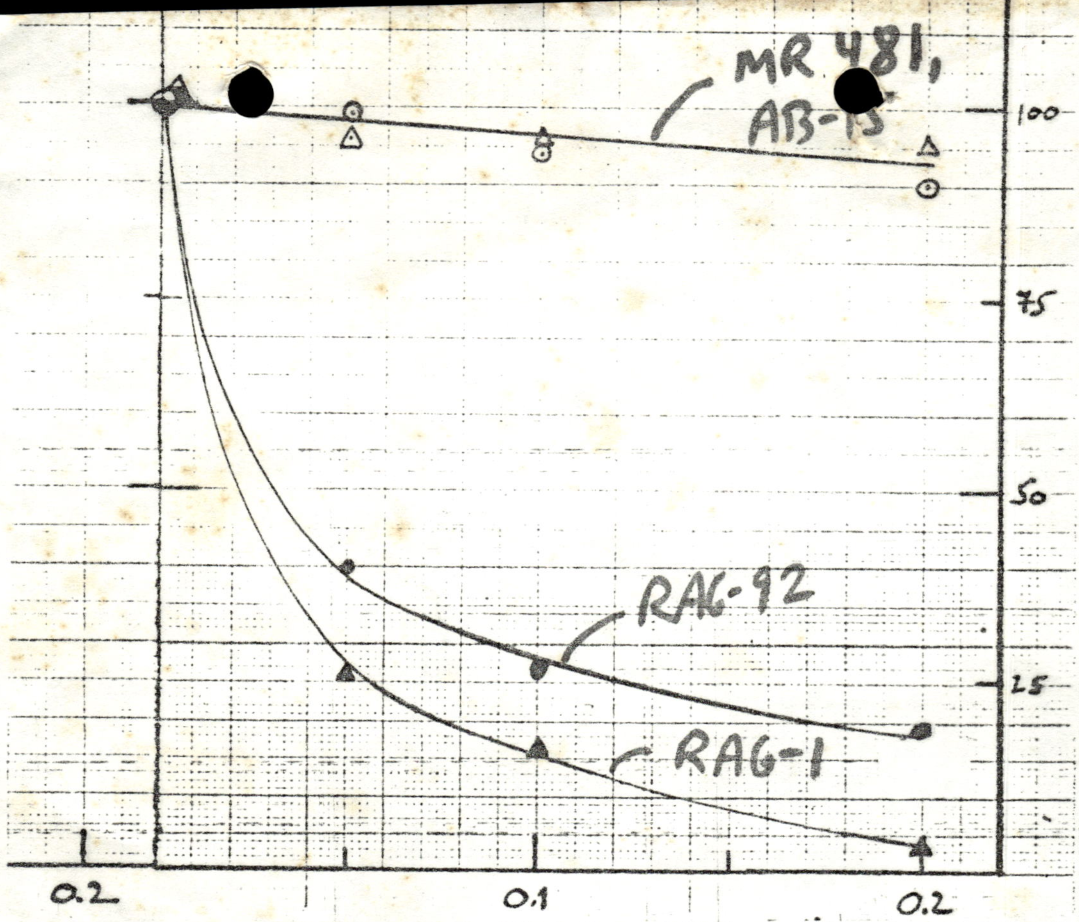

EtOH gram.

1.35-1.55

19/8/81

26/6/81

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Cell Surface Properties of RAG-1 STRAINS related to adherence and growth on H

Strain	% Adherence to 0.2 ml HD	% Adh to 0.2 ml XY	Growth on HD vapour	Growth in HD tube	Thin Fimbriae (EM)	Agglutination ($T_{1/2}$)	Phage Sensitivity			
							215H	ø11	AP-3	ø1
RAG-1	96	95	++	+++	++	~22	S	S	S	R
MR-481	2	18	++	—	-	>>48	S	S	S	R
RV-1	14	48	++	+	+++	<6	S	R	S	R
RV23-4	57	92	++	++	+++	<6	S	S	R	S
RAG-92	100	n.d.	++	+	++	~22	S	S	S	R
AB 15	95	n.d.	++	—	—	>>48	S	S	S	R

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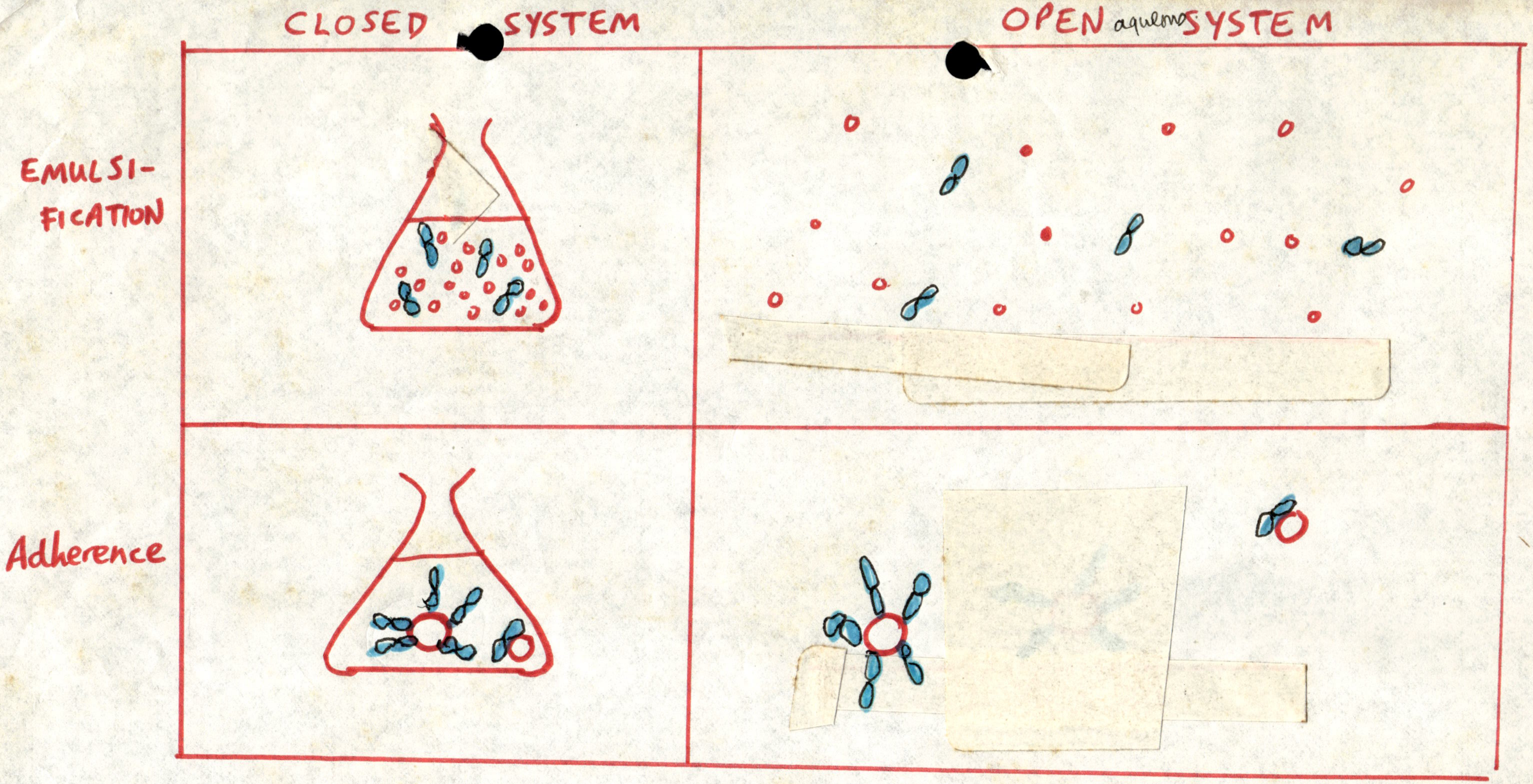
CLOSED SYSTEM
OPEN aqueous SYSTEM
EMULSI-FICATION
Adherence

CONCLUSIONS:

1. Adherence to hydrocarbons is a measure of cell surface hydrophobicity, rather than a specific property of hydrocarbon-degrading bacteria.
2. Adherence to hydrocarbon is crucial for growth under conditions of (i) low initial cell density, and (ii) limited emulsification
3. Thin fimbriae play a role in adherence of RAG-1 to hydrophobic surfaces and growth on hexadecane.

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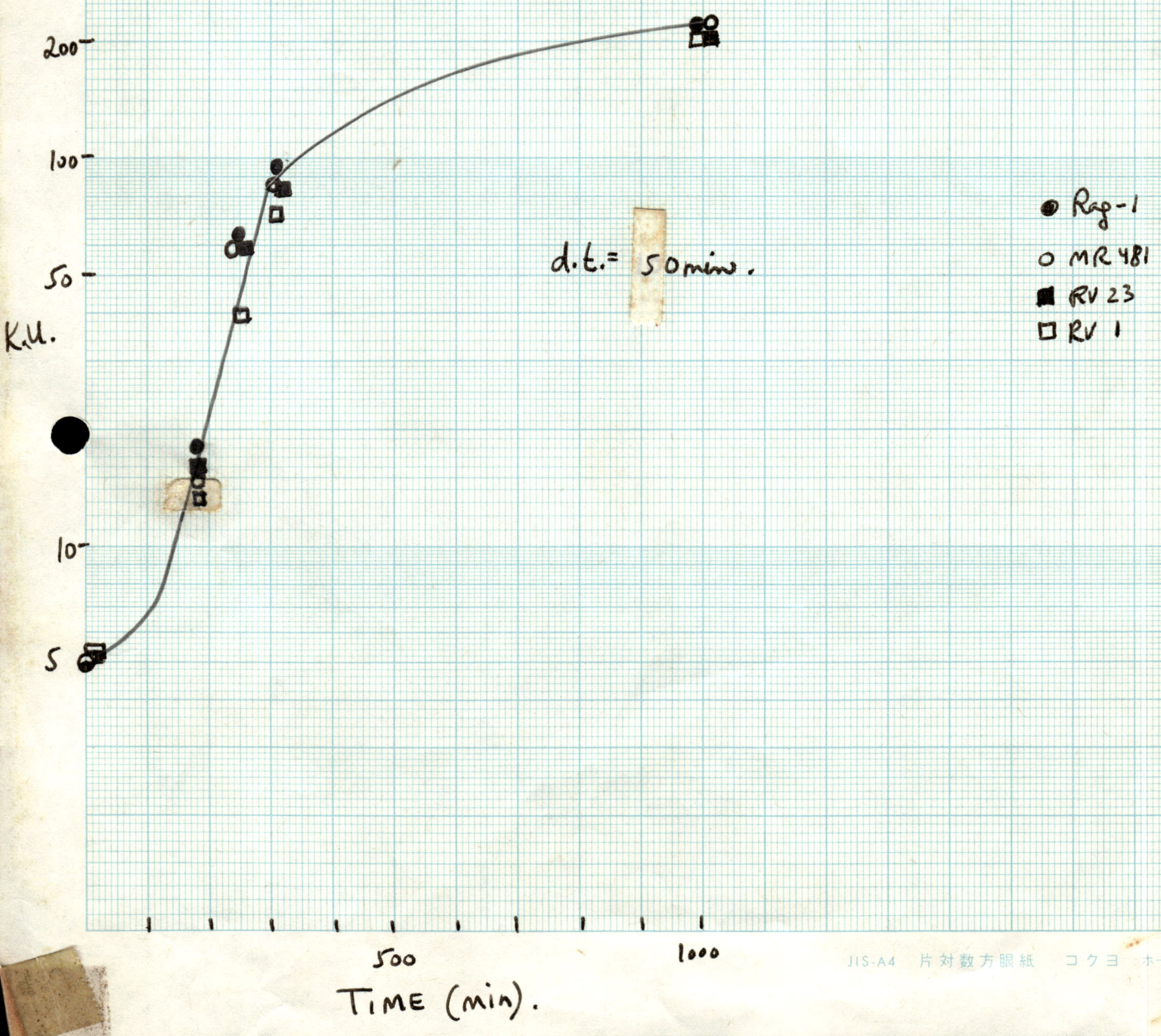
Fig. 23.

Growth of Rag-1, MR481, RV1 and RV 23-4 on 0.2% acetate 10/11/81



d.t. = 50 min.

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Sonication of RAG-1 and MR-481

31/5/81-2/6/81

RAG-1 and MR-481 cells (800 ml each) were grown in 4l. flasks on Brain Heart Infusion and washed 3x in H_2O following growth to stationary phase (22h.). Cells were resuspended in H_2O to a concentration of

RAG-1 : 43 mg/ml } dry wt.
MR-481 : 45 mg/ml }

7.5 ml. Cells were sonicated at 0°C at 70 Watts and samples taken at the following times:

Time (min)	Sample vol. ml.
0	1.0
½	1.0
2	1.0
4	1.0
6	3.5
	7.5

Turbidity was measured in each sample. Sonicates were frozen, thawed, and spun at 10000 r.p.m. for 30 min. Pellets were resuspended in PUM for adherence to hexadecane. Sups were centrifuged again in the eppendorf and frozen.

Results -

1. Turbidity (K.U.)

TIME (min)	RAG-1	% of control	MR481	% of control
0	1,7745	100	19,773	100
½	14,027	79	15,640	90
2	8,957	50	10,309	52
4	5,577	31	5239	26
6	3,380	19	3380	20

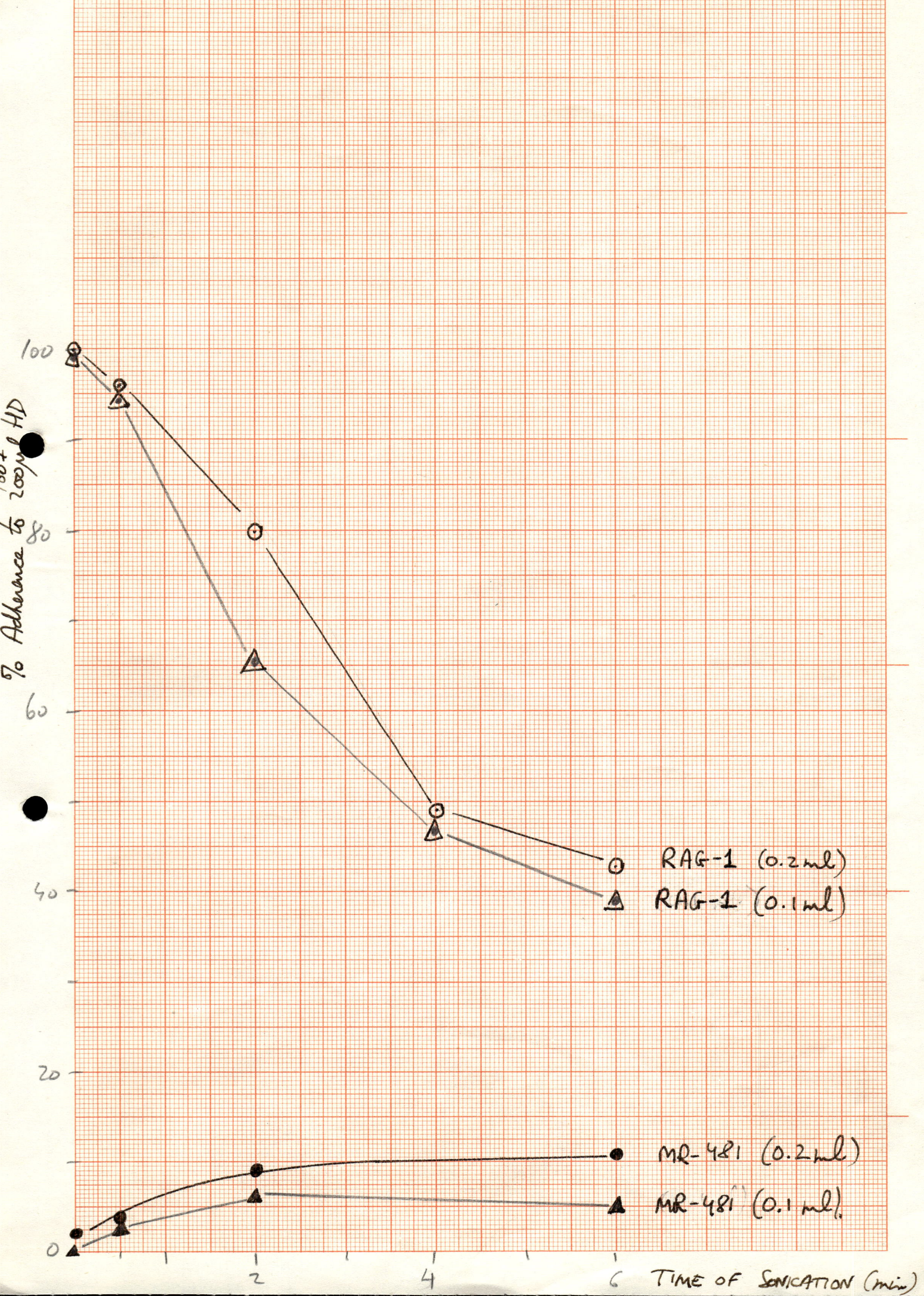
2. Percent Adherence to HD

TIME	RAG-1 (ml HD)				MR 481		
	0.1	0.2	0.5	INITIAL O.D	0.1	0.2	INITIAL O.D.
0	99.5	100.0	95.0	1.244	0.4	1.9	1.143
½	93.9	96.3	98.4	1.273	3.3	4.1	1.337
2	65.6	80.6	71.4	1.295	7.3	8.9	1.248
4	47.4	48.9	44.7	1.331	-	-	-
6	39.7	43.4	52.7	1.227	4.8	11.4	0.989

Conclusions - While sonication for 30s has little effect on adherance, prolonged sonication (as described by Wadstrom - Current Microbiol.) does have significant effect in lowering adherence of RAG-1 cells to HD. Similar treatment of MR-481 caused a slight (not very significant) rise in adherence of mutant cells.

Adherence to Hexadecane (0.1 and 0.2 ml) as a Function of Duration of Sonication

1/6/81



Decrease in Turbidity as a Function of Duration of Sonication 1/6/81

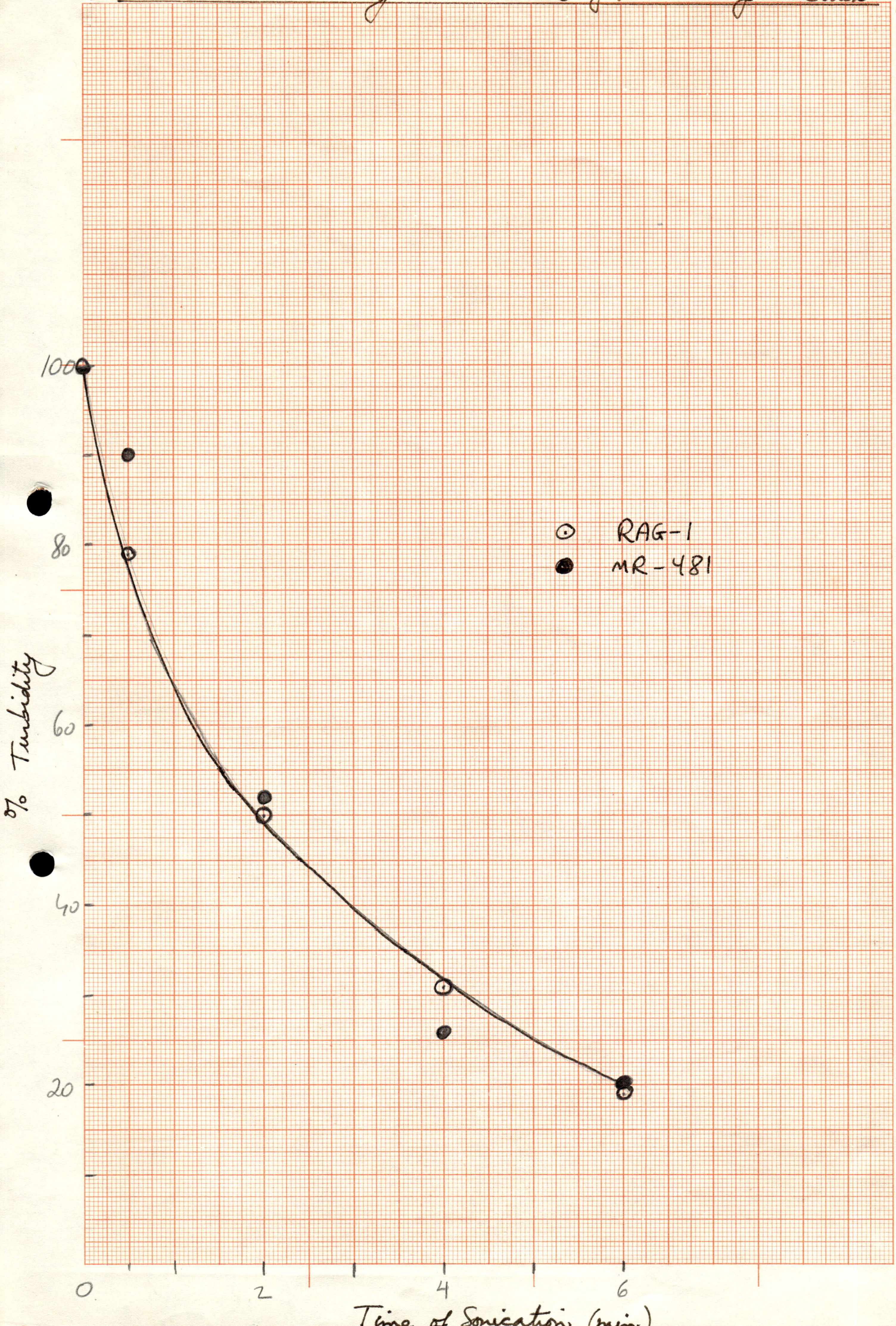


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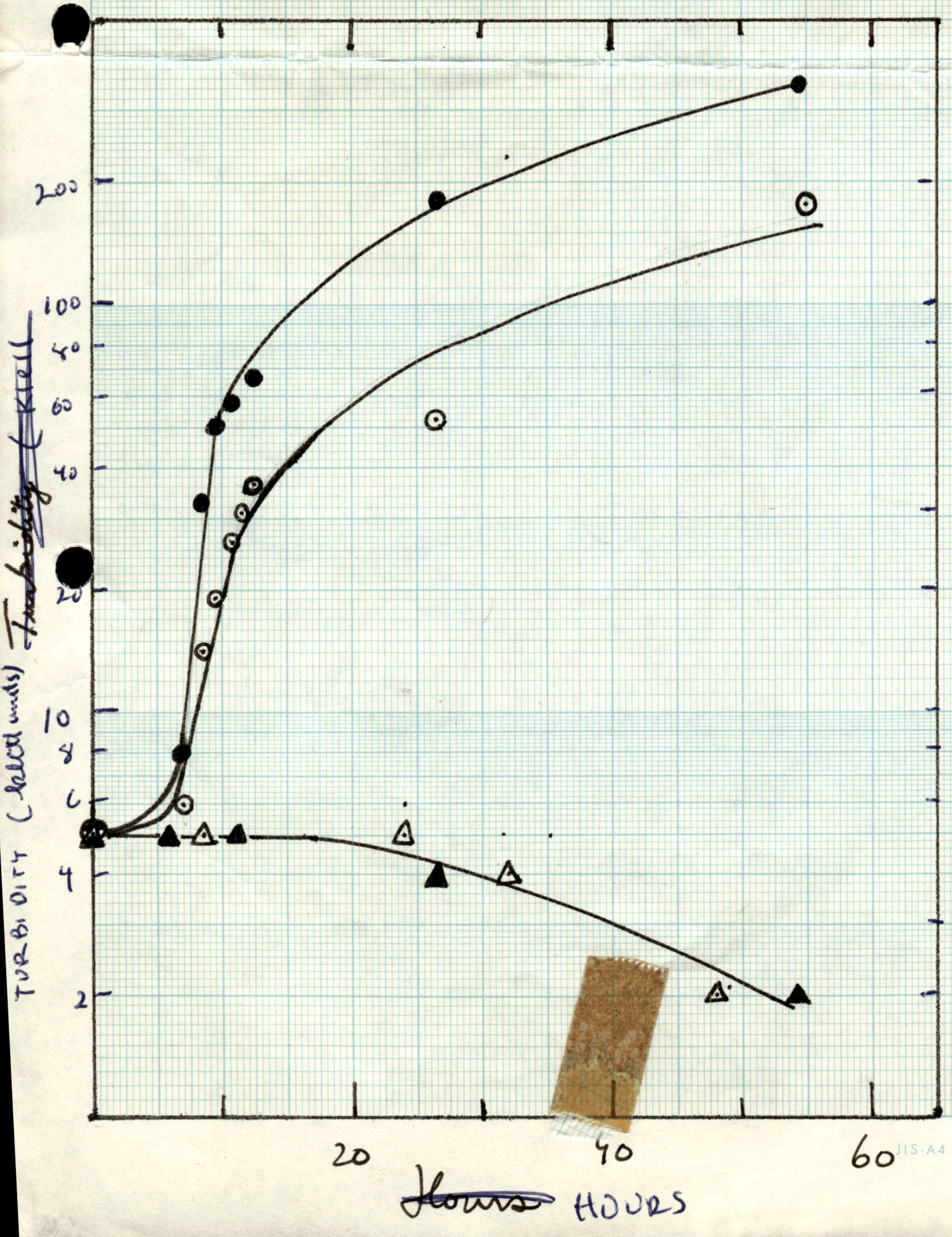
FIG. 5

Growth of RAG-1 and non-adherent mutant on HD in the presence of emulsan 9/2/81



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On board: neutral lipids table
diauxy curve.

1. Acinetobacter

formerly Micrococcus cerificans. grows on C_{10}-C_{20} alkanes alk-1-enes. Growth on alkanes and alklene causes induction of two systems, perhaps related.

2. Membrane induction

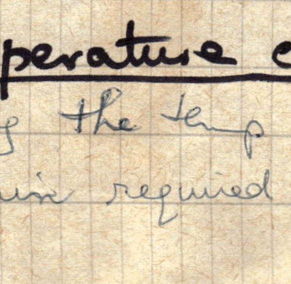
rich in lipids, low density. Isolated by lysozyme treatment, followed by density gradient.

3. ~~Hydro~~ Hexadecane inclusions

can be seen, sometimes connected to intra-c membrane. may be artifact.
Isolated by French pressure. band out at top when centrifuged. passed through Sepharose column - two bands: soluble protein and inclusions. contain very small amount of hexadecane - could this be artifact. No bilayer limiting membrane. mostly neutral lipid.

4. ~~Auxo~~ Diauxic Growth

for example cells [illegible] given limiting acetate 5mM and excess hexadecane. Only when acetate exhausted do we find induction of systems.



5. Temperature effect:

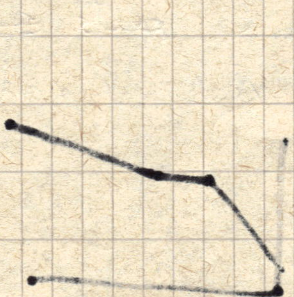
lowering the temp from 25°-20° lowers the min chain required for Carbon source C_{12}-C_{10}

1. Acinetobacter

2. Membrane induction

3. Hydro Hexadecane inclusion.

4. Auto [illegible] Growth



5. Temperature effect:

W165, 23128

Modrzakowski, M.C., R.A. Makula + W.R. Finnerty
J Bac. 131 92-97 1977
metabolism of n-dioctyl ether.

↙ ↓

8 n-octoxyl octanoic acid 2 n octoxyl acetic acid.

growth occurs at expense of 6-C atoms of dioctyl ether

A. G. McLee & S. L. Davies
- used slide cultures to show that growth of a hydrocarbon utilizing Torulopsis sp (yeast) occurred only when in contact w/ n-alkane droplets.

yeast grow on the droplets but don't emulsify – quite the contrary – when hexadecane is provided in highly emulsified n-alkane no yeast stuck to it.

Finnerty W.R., Hawtrey, E. and Kallio, R.E
Z. allg. Mikrobiol 2, ~~4~~ 169 (1962) 263-266?
(not in library.

McAuliffe (1963) Nature 200, 1092. } solubilities of alkanes in water
(1969) Science 163, 478

(25°-20°)
lowering the growth temp could lower ~~the~~ the min chain length for growth → how explain?
toxicity – BS

Finnerty ~~et al. 1964.~~
WR and Kallio RE (1964) J Bac 87, 1261

cetyl palmitate; & C_1^{14} labelled hexadecane fed.

Seminar on Hydrocarbon Inclusions

Micrococcus cerificans — newname Acinetobacter

(alk-1-enes)
Growth on paraffin + olefinic hydrocarbons led to cytoplasmic modification,
Can see intracytoplasmic hydrocarbon inclusions
ON NBYE, don't see anything

Figs 2 and 3 show pictures — OsO_4 fixation followed by dehydration in rising EtOH concentration,
next propylene oxide, finally EPON.
p79 note diff between A and C (grown on C_{16} vapours)

Id of inclusion components:

1. GLC: ~~alcohol~~ cells washed 5x with H_2O, cells then alcohol extracted
GLC showed the same hydrocarbon that the cells were grown on.

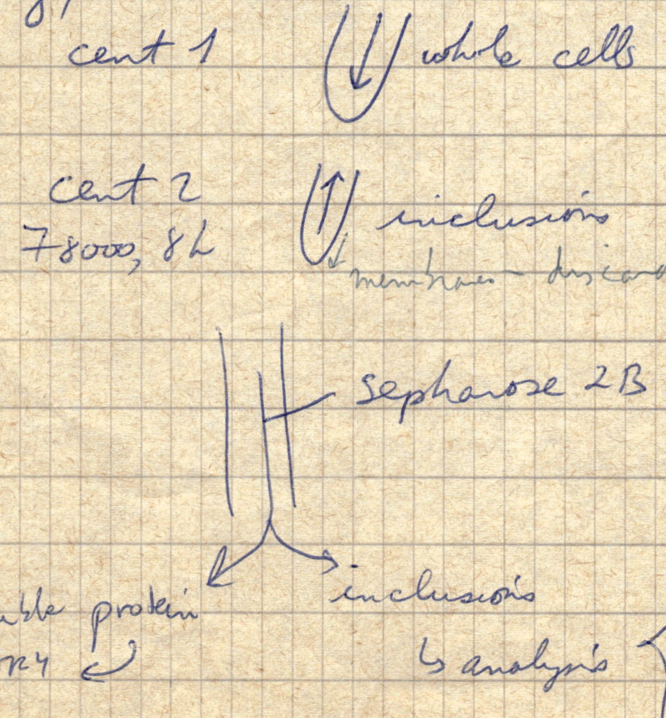
2. X ray diffraction analysis:
cells washed many times and lyophilized cooled to −10° suddenly, transforming the hydrocarbon to crystalline form, enabling X-ray diff analysis
Same problem: cannot prove that this is not artifact.

no poly β hydroxys in either grown cells

The accompanying article shows the development of "intracellular" membrane system which may or may not be physically associated to the inclusions cells fixed with glutaraldehyde (to see the membranes better). These membranes were later isolated and found to be rich in lipids, and of low density.

Two years later, another pair of articles:
(Arch Microbiol).
(2nd will come first here).

Hexadecane inclusions isolated:
cells washed w/TRIS buffer this time (instead of water) + passed through French press, chilled, at 4,500 kg/cm^2


cent 1
whole cells
cent 2
78000, 8h
inclusions
membranes - discard
Sepharose 2B
Soluble protein
Lowry
inclusions
↳ analysis

20 μm $C_{16}H_{34}$
~~mostly~~ per mg protein
ie; $\frac{4.24 \text{ mg } C_{16}}{\text{mg prot}}$

FREEZE ETCH: Show figure 9 which indicates continuity of IM w/CM.

Rel conc. of neutral lipids in $C_{16}H_{34}$ inclusions

	%
wax ester	50.5
free fatty acid	5.8
free fatty alcohol	17.6
triglyceride	5.7
diglyceride	6.9
monoglyceride	63
HEXADECANE	12

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5/8/80

Isolation of Non-Adherent Mutants of RAG-1

Non-adherent mutants of RAG-1 were obtained by the following technique - 50 ml. of a stationary phase culture of RAG-1 ~~were~~ in Nutrient broth (30°C, aeration) were harvested and resuspended in 5 ml PUM buffer. 2-5 ml octane was added and following mixing and phase separation the bottom phase was transferred to another test tube. 2 ml octane was added and the process continued until no cells were microscopically visible in the aqueous phase. 50 ml. of ~~nb~~ nutrient broth were inoculated with the final lower phase and when the cells reached stationary phase this whole process was repeated. When the fourth nutrient broth culture was spun down, resuspended in PUM and octane added, turbidity remained in the lower phase even after several mixings and transfers. The aqueous phase was streaked out and three colony-types were observed:

481-B. Colonies resembling RAG-1 on NB and EtOH plates

482-S. Similar colonies, but slower growing. After several days incubation at 30°, dense inner circle surrounded by translucent mucoid growth.

483-T. Tiny colonies on NB and EtOH. Large colonies ~~cc~~ on LB plates.

Microscopic observation - 481-B and 482-S appear similar to RAG-1. While 483-T is a diplococcus, it appears in irregular clumps and does not resemble RAG-1 in appearance, Following isolation of these three distinct types, they were grown 18 h. in brain heart infusion broth (bhib) at 30°C w/ shaking, and assayed for adherence, with RAG-1 as control.

Results - (% adhering cells; initial O.D. in parentheses)

Hexadecane vol. (μl)	RAG-1 (1.563)	481-B (1.375)	482-S (1.576)	483-T (1.479)
20	97.4	0	62	0
50	95.6	0	~~64~~ 69	0
100	94.6	0.6	~~64~~ 65	0
200	—	3.4	68	0

Following the adherence assay the (same) cells were checked for (1) phage sensitivity and (2) agglutination with antibody

Results -

Phage sensitivity

(top agar method§; repeated twice) STRAIN:	ϕ2	ϕ3
RAG-1	S	S
481-B	S	S
482-S	R	S
483-T	R	R
312	S	R
201	R	S

Agglutinability - Assayed by microscopic observation; three concentrations of antibody; compared with cells alone and cells + serum:

Strain	Agglutinability by	
	"95"	"5"
RAG-1	++	++
481-B	$++^{L}$	+++
482-S	$+^{L}$	++
483-T	—	—

Cells from these liquid culture were streaked onto plates; then bhib cultures were ~~and~~ reinoculated from these plates and adherence assayed again:

HEXADECANE VOL (ml)	RAG-1 (1.322)	481-B (1.351)	482-S (1.540)	~~483-T~~
50	98.5	0	30	
200	—	27	22	

Conclusions - These experiments established that 481·B and 482·S are indeed mutants of RAG-1 and are deficient in ability to adhere to hexadecane ~~and~~ when grown under conditions which promote adherence in the wild type. 481-B appears to be wholly deficient and 482-S partially deficient. 483-T appears to be a contaminant.

8-184

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Table 1. Effect of Periodate Treatment and Sugars ~~Addition~~* on adherence of ~~late-exponential~~ RAG-1 ~~cells~~ to Epithileal cells.

Inhibitor used ~~Addition~~**	Adherence (% of control)	6
None	100	100
oxidation of epithelial cells ~~None (periodate treated)~~*** with sotium metaperiodate *	40	0
D-mannose	40	10
~~β~~ methyl~~α~~-D-mannosite	54	5
~~β~~-D glucose	98	>90
methyl-β-D-glucoside	86	~~>90~~
D-glucosamine	107	n.d.
N-acetyl-D-glucosamine	116	85
D-galactose	88	99
methyl-α-D-galactoside	112	>90
maltose	100	>90
L-arabinose	100	n.d

* Adherence was performed in the presence of various sugars at a final concentration of 2.5%

** In the control 43% of the epithelial cells ~~scaed~~ scored ~~to~~ had 100 or more attached bacteria

*** Epithelial cells were incubated 15 min. in the presence of 10 mM sodium meta periodate as previously described (6).

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Growth of RAG-1 and Mutant MR-481 on Hexadecane

10/2/81

Rag-1 and MR-481 were harvested in stationary phase following 18 h. growth in brain heart infusion broth, washed carefully twice in PUM and resuspended to 102 KU (A_{540}). Green Filter
Cells were diluted $\frac{0.2}{4.2}$ into PUM. To 4.2 ml aqueous cell suspension (.5ml hexadecane) were added 10% (W/v) hexadecane and test-tubes were incubated at 330RPM in a New Brunswick gyrotory water bath shaker at 30° c. Growth was followed by measuring turbidity in the lower aqueous phase.

Additions:

Rag-1

R-1, 2, 3 → cells + HD

R-4 → cells + HD + emulsan
at time 0
(50μg/ml final, S-5-W)

R-5 → cells + emulsan (-HD)

R-6 → cells + 0.2% Na-acetate

MR-481

B-2, 4 → cells + HD

B-5 → cells + HD +
emulsan at time 0

B-3 → cells + HD +
emulsan at 10h

B-1 → cells + HD +
emulsan at 24h.

B-6 → cells + emulsan (-HD)

B-7 → cells + acetate 0.2%

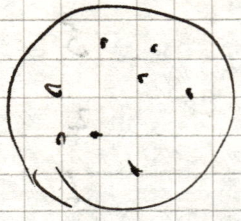
Initial viable counts

Rag-1	5.2×10^7
MR-481	6.5×10^7

Results

1. Growth on 0.2% Acetate:

Time (h).	K.U. corr	
	Rag-1	MR-481
0	5	5
1.25	1	2
2.5	7	6
2.9	10	8
3.2	18	16
3.5	17	16
4	36	24
4.3	38	34
4.6	45	44
5	55	58
5.25	65	65
5.5	71	74
6	86	86
7	105	110
8.5	146	164
9.5	170	190
10.7	180	217
11.3	188	254
12.5	190	251
24	160	184



2. Growth on Hexadecane (10% v/v).

					K.U. (corr)						
Time (H)	R1	R2	R3	R4	R5	B1	B2	B3	B4	B5	B6
0	5	5	5	5	5	5	5	5	5	5	5
2.5	6	3	4	2	4	5	4	5	10	5	4
3.5	3	0	4	-2	4	3	2	5	10	3	6
5	9	6	6	2	6	4	5	8	9	4	6
6	11	6	4	4	5	4	6	7	9	4	4
7	17	7	7	8	3	4	6	8	9	6	4
8.5	34	13	9	33	4	3	5	7	10	14	5
9.5	45	17	11	50	5	4	6	7	10	19	4
10.7	51	22	12	57	1	3	4	7	10	26	4
11.3	55	23	15	62	5	2	5	6	10	31	4
12.5	58	32	19	66	5	3	6	7	8	36	3
24	130	62	50	144	2	3	6	22	8	50	5
26.5	168	72	55	178	4	4	6	28	9	52	5
32	210	92	61	230	1	7	3	39	10	63	4
35.25	230	126	67	290	0	8	5	56	8	68	2
48	245	185	164	325	0	21	3	178	8	93	2
54.5	240	200	225	350	2	51	4	260	10	178	1

LAG (17 K.U)	7	9.5	12
~~100~~ 60 KU	+5.5	+14	49
60 − 168	14	16	15

3. Reasons for the Results -

- careful washing of the cells
- good ventilation
- pipetted hexadecane with glass pipette
- started at 5 K.U.

4. Adherence - After 54 h. growth, cells of R-1, R-4 and B-5 were centrifuged at 4°C and, washed twice in PUM and resuspended to ~1.4 O.D.$_{400}$ Adherence to 200µl hexadecane as usual.

Results:

Sample	Initial O.D.	Following mix w/200µl	% adherent
R-1	1.416	0.094	93.4
R-4	1.396	0.052	96.3
B-5	1.426	1.361	4.6

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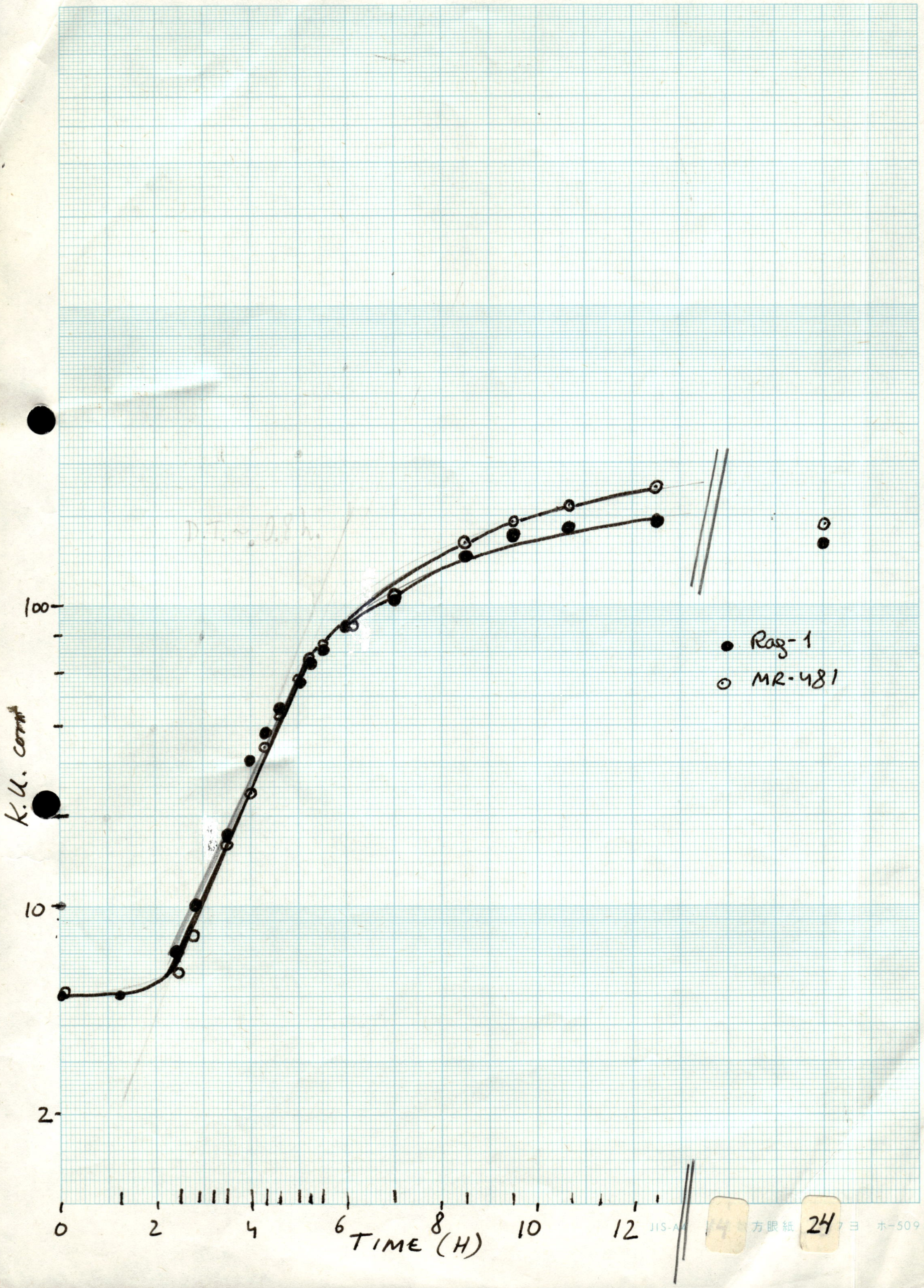
Growth of Rag-1 and MR-481 on 0.2% Na Acetate 9/2/81



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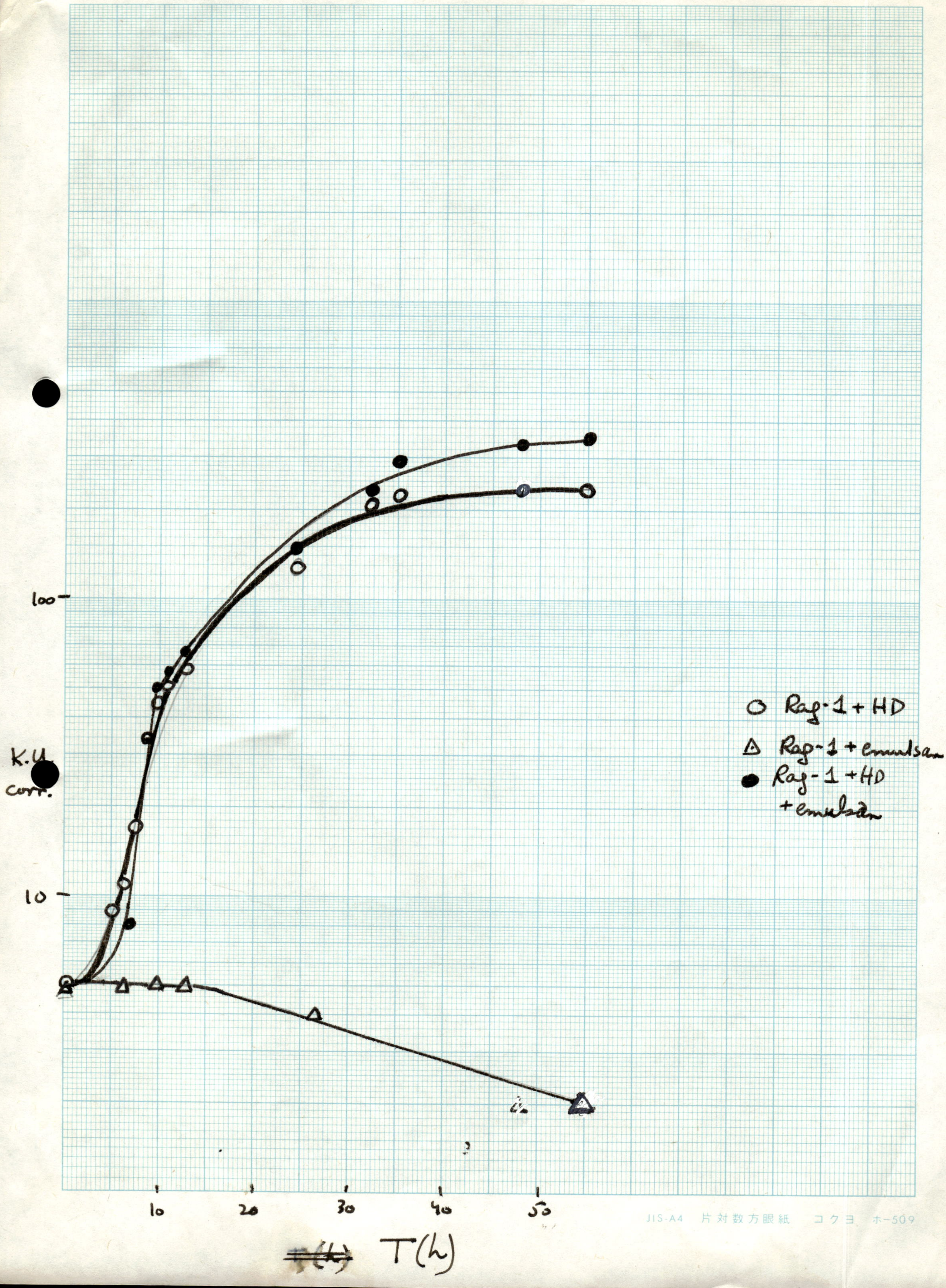
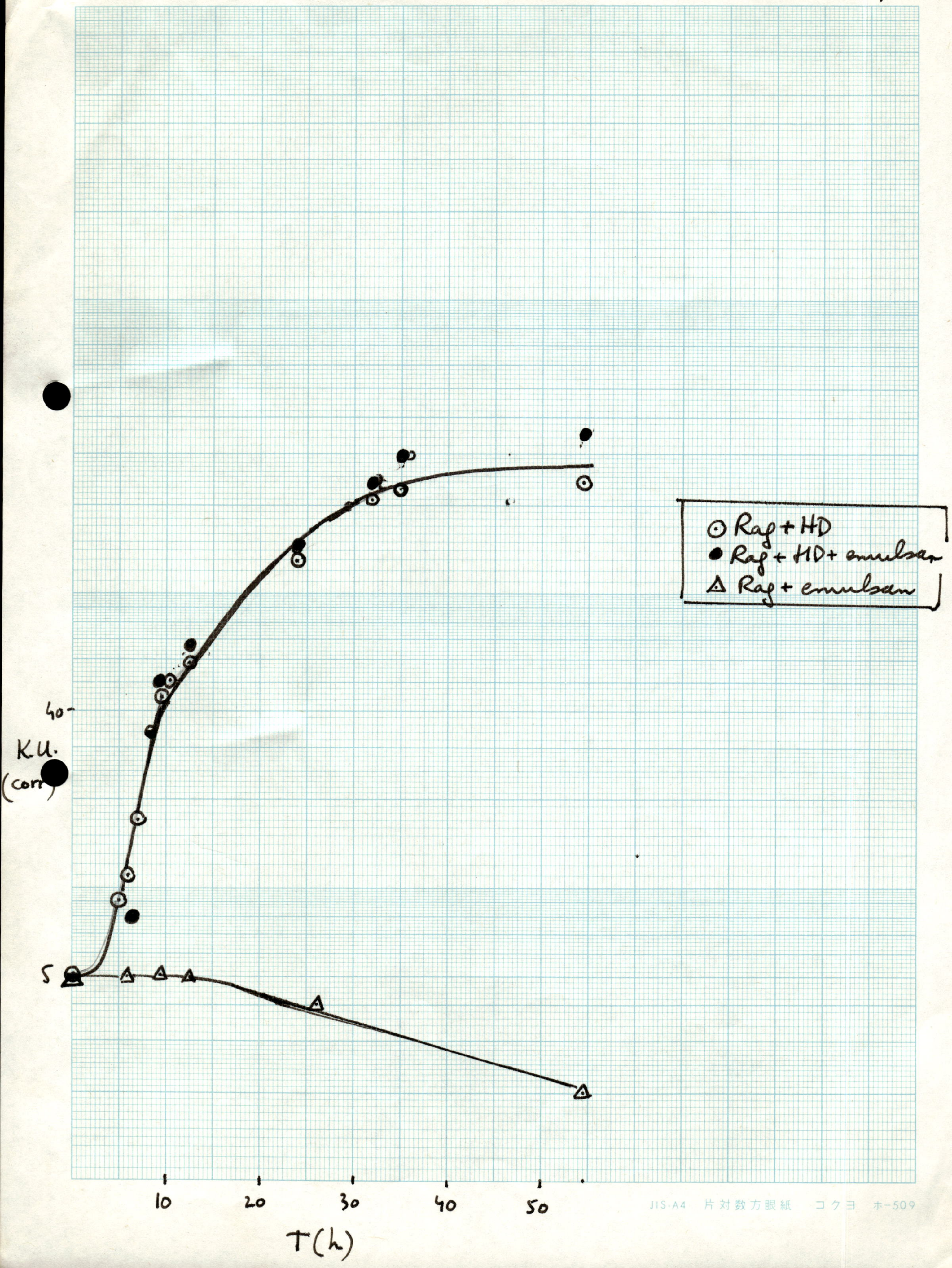
Growth of RAG-1 on Hexadecane 9-10/2/81



JIS-A4 片対数方眼紙 コクヨ ホ-509

9-10/2/81



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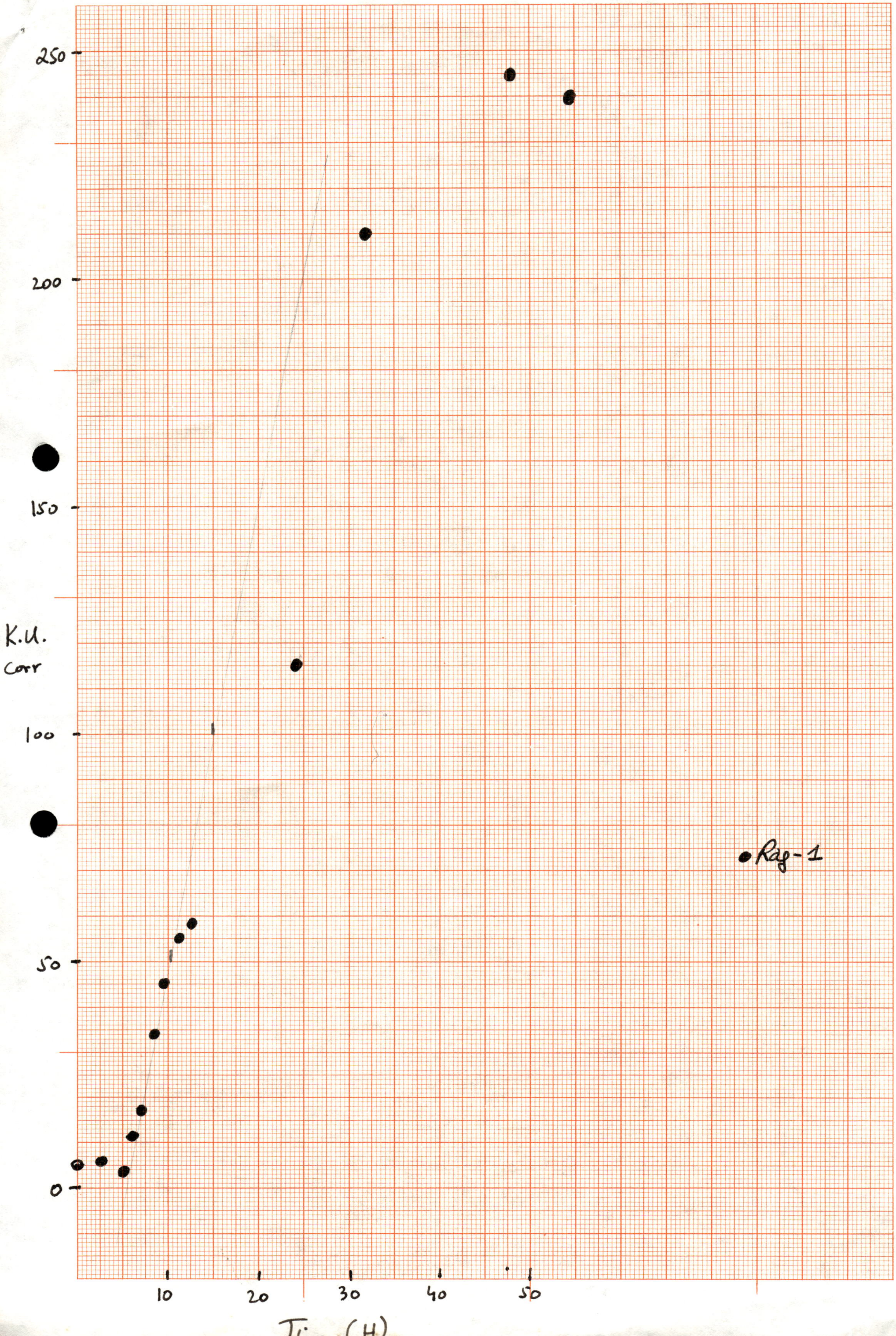
250
200
150
100
50
0
K.U.
corr
10
20
30
40
50
Time (H).
Rag-1

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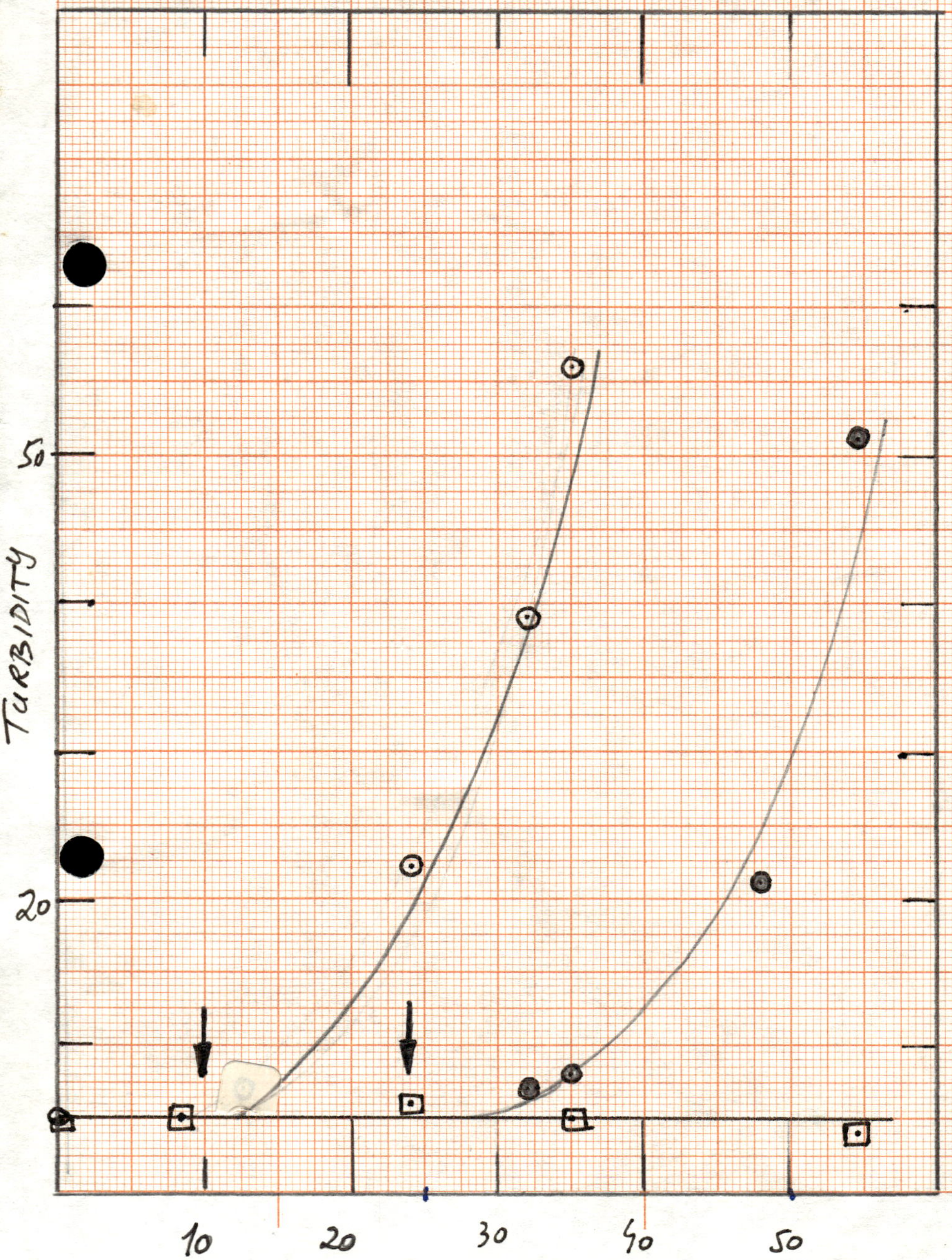
Induction of Growth of MR-481 on Hexadecane by Addition of Emulsan. 9/2/81



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Growth of Mutant MR-481 on Hexadecane in the Presence of 50μg/ml S-5-W Emulsan

9/10/2/81

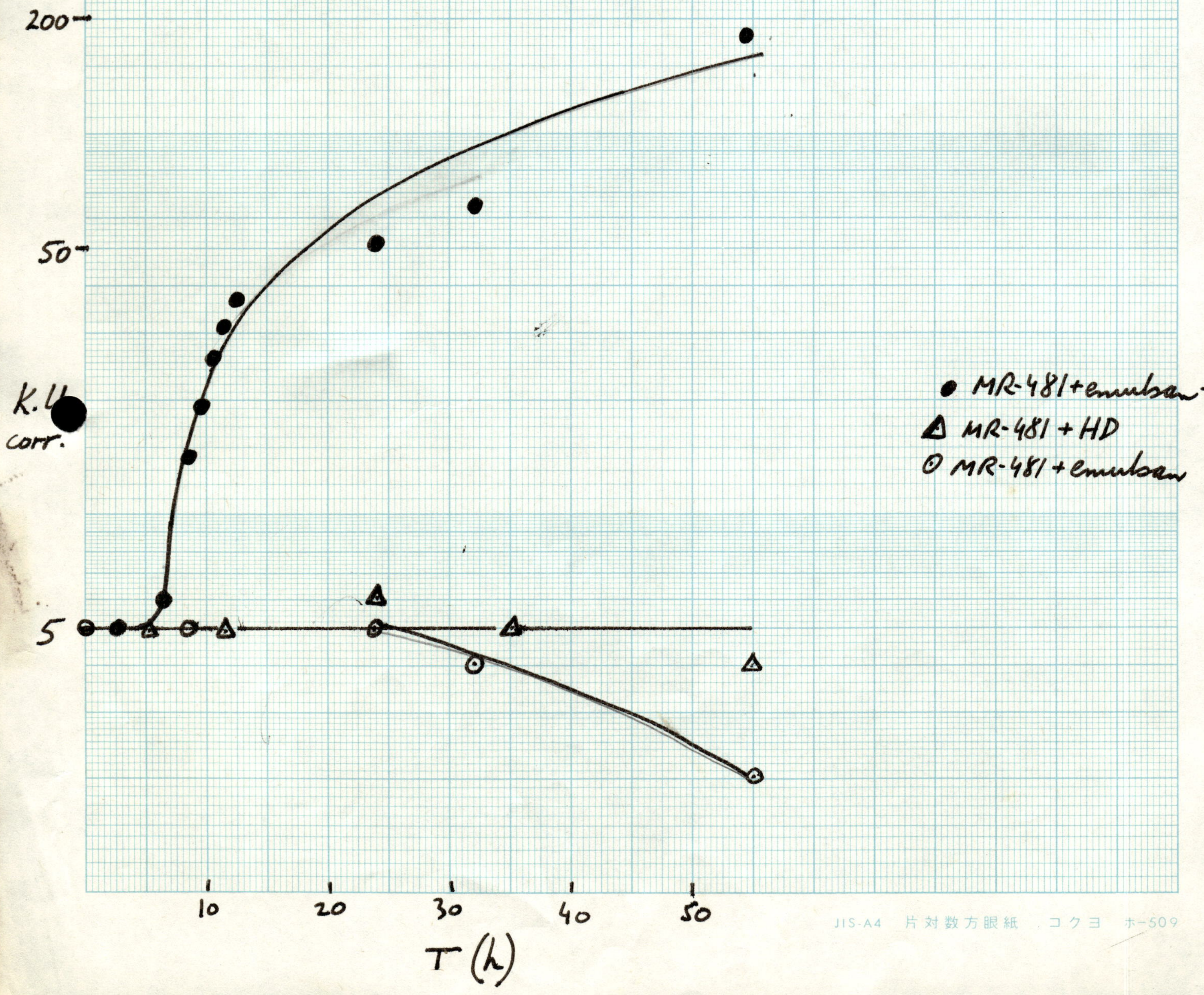

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Fig. 6.

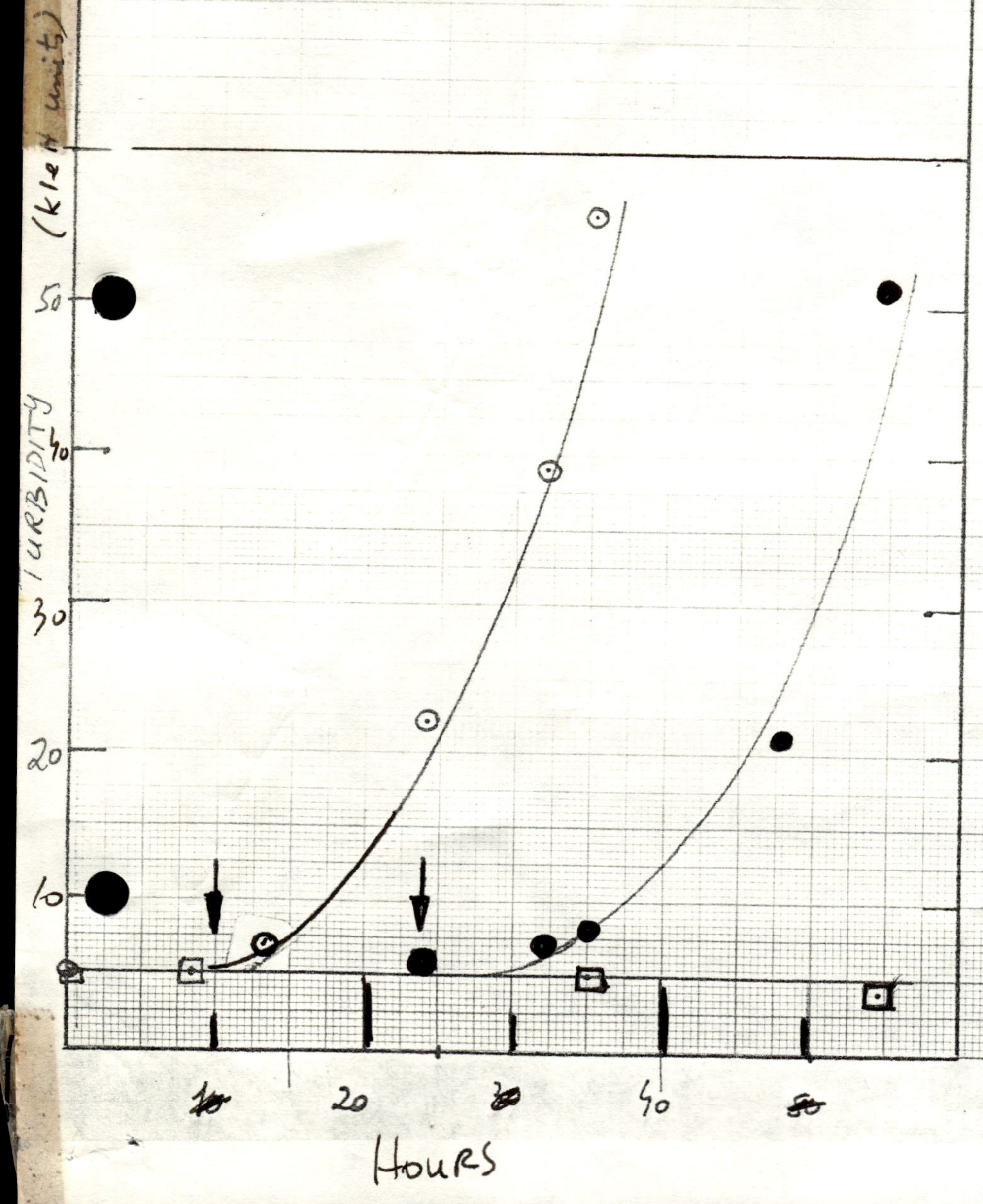


FIG. 2

Cogrowth of RAG-1 and non-Adherent Mutant
on 0.2% sodium acetate

9/2/81

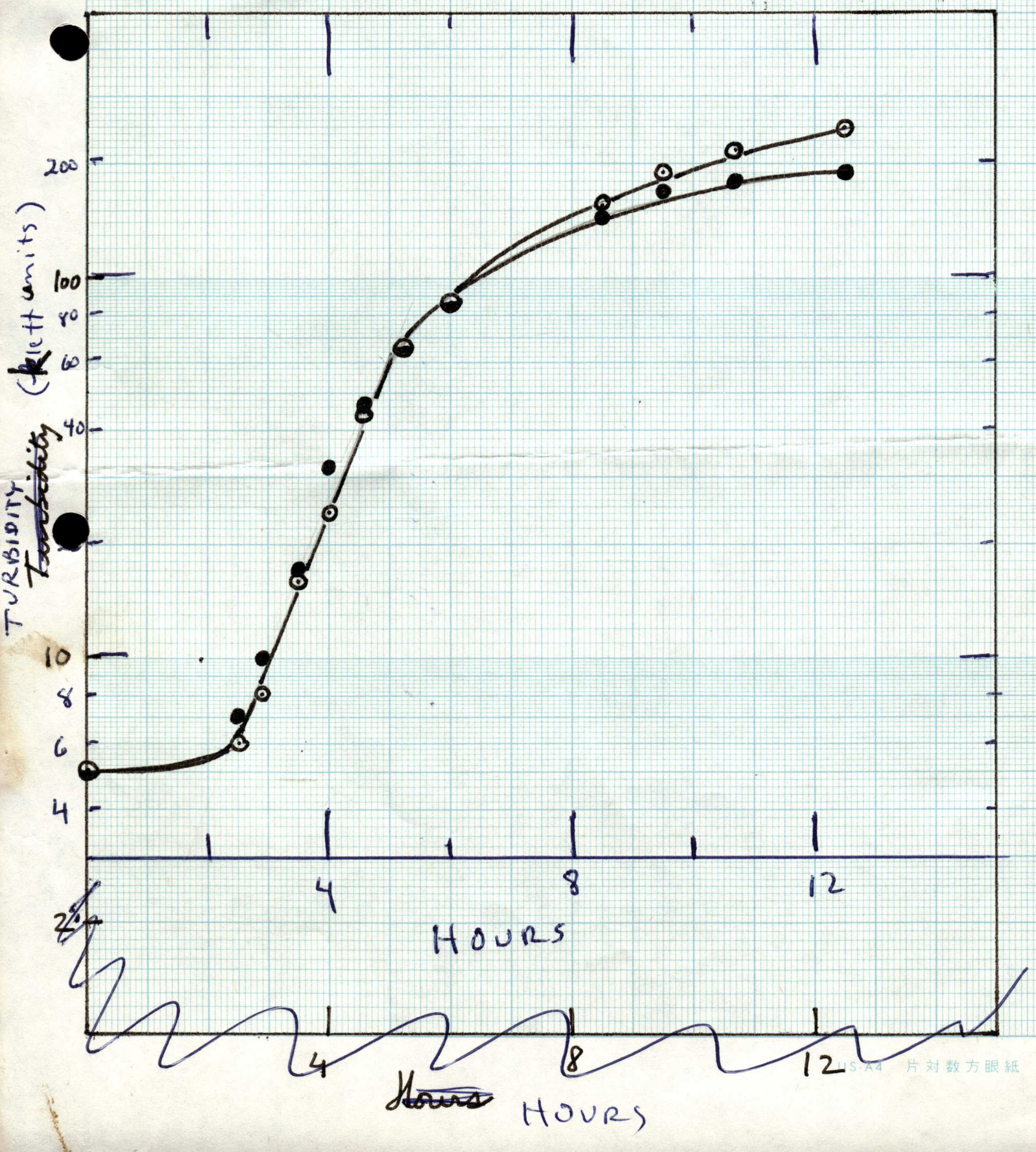
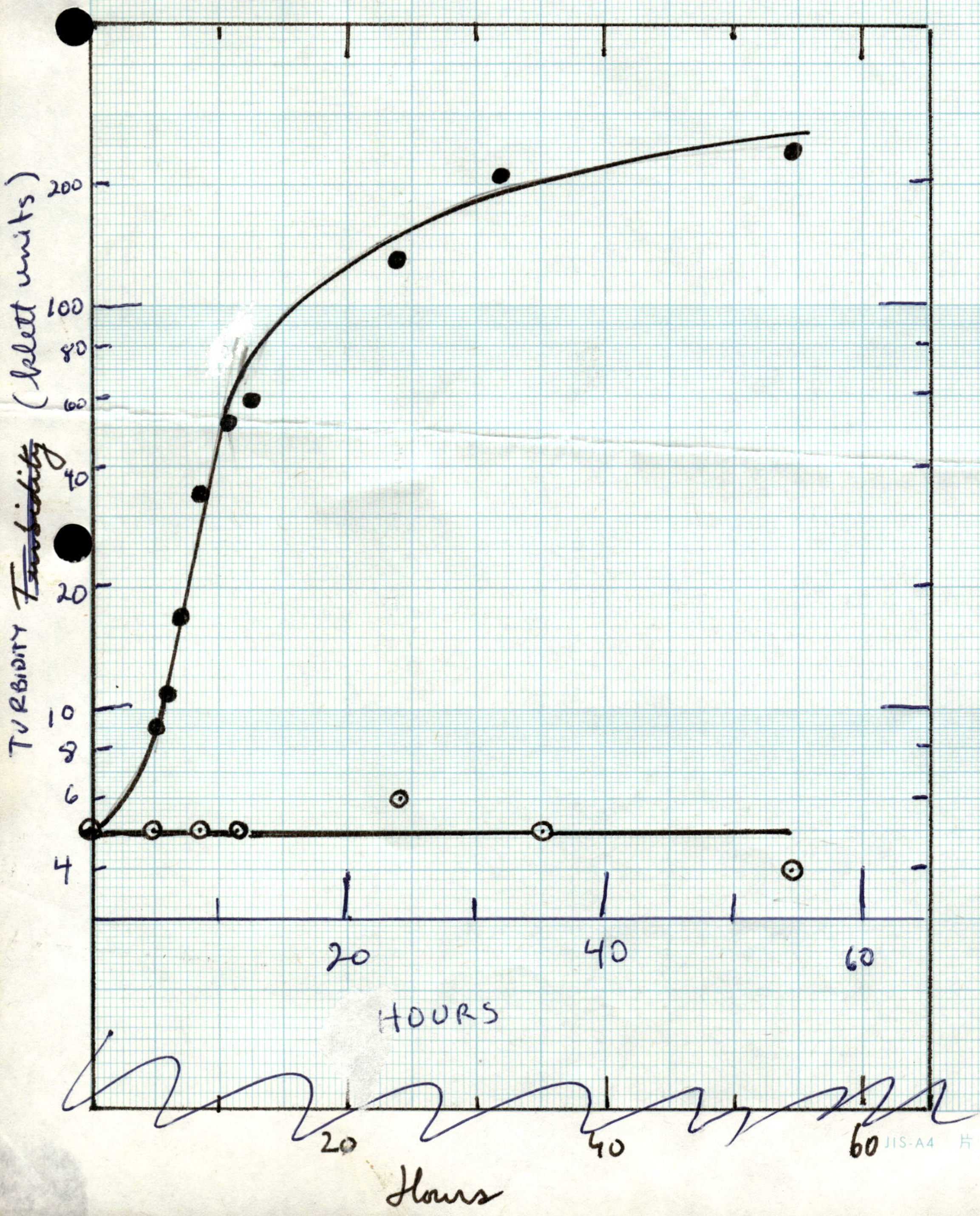


FIG. 3

Growth of Rag-1 and non-adherent mutant on HD

9/2/81



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supplemented by casamino acids (Fig 1.), table.

Medium ~~Table~~	Phase	K.U.	% Removed by 20 µl HD.	~~8~~
NB	early log 6h	~~14~~	0	
	late log 7.5	~~63~~	74	
	stat (18h)	~~130~~	87	
EtOH + cas	early log (6)	~~30~~	0.8	
	late log (11)	~~150~~	~~11~~ 13	
	early stat (18h.)	~~400~~	32	
EtOH.	~~early log~~ exp	50 ~~[illegible]~~	~~[illegible]~~ 13	
	~~early lat~~ stat (24h)	(320 uncorr)	30	
HD	24h.		71	
	54h.		87	

Very young cells harvested from NB and Et + cas cultures showed no significant affinity towards hexadecane. (Fig. 1, Table 1). Adherence to hexadecane ~~appeared to develop with~~ was found to ~~develop along~~ increase with culture age in all media ~~test~~ studied. However, late log and stationary cells ~~[illegible]~~ were found to adhere to ~~the~~ the hydrocarbon. The ~~most~~ greatest ~~differences~~ increases ~~is~~ were observed during growth on nutrient broth; ~~Whereas young cells sho~~ 20 µl of HD removed 74 and 87% of the cells of late log + stat. culture, respectively, whereas only 5% of the cells were removed by 200 µl HD.

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19/6/81

Adsor Agglutination of RAG-1, MR-481 and 15 by IG-UB antibodies

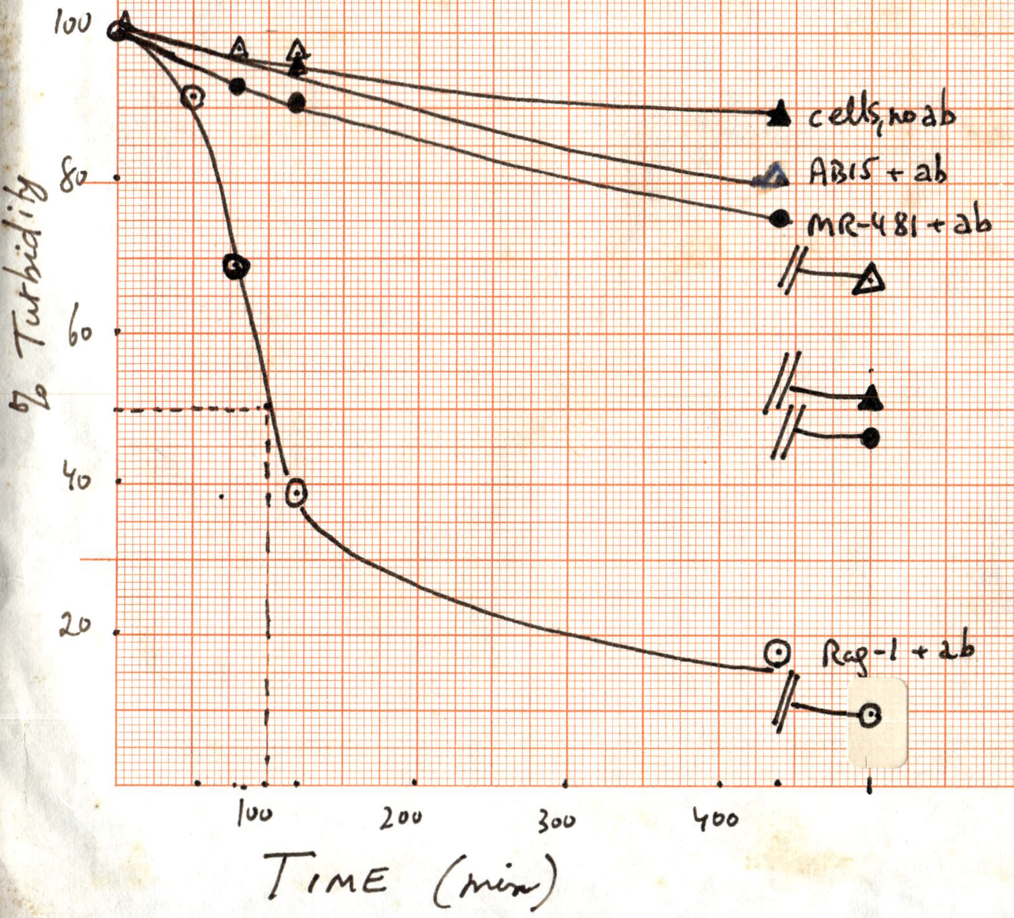
Cells were grown to stat. phase in brain heart, washed and resuspended in PBS to $A_{400} \sim 1.3$. To 700 µl cells, 3 µl of IgUB antibodies were added and agglutination followed turbidimetrically as a function of time, at room temperature

Results

Sample	Initial A_{400}	% Turbidity					
		25'	55'	80'	120'	440'	2880'*
RAG-1	1.331	98	97	97	95	90	47
MR-481	1.343	99	96	97	94	89	55
AB-15	1.288	99	96	96	96	90	51
RAG-1 w/ab	1.354	99	92	69	39	18	10
MR 481 w/ab	1.382	99	96	93	91	75	46
AB-15 w/ab	1.343	99	97	97	97	81	67

* In fridge

Agglutination of RAG-1, MR-481 and AB-15 by IgUB (nonadsorbed antibodies).



19/6/81

Adherence of RAG-1, MR-481 and AB-15 to Hexadecane following growth on B.H.I.

Cells were grown to stat. phase on brain heart, washed and resuspended in PUM to A_{400} ~1.5-1.6

Adherence to hexadecane as always.

Results -

Sample	Initial A_{400}	% Adherence		
		50µl	100µl	200µl
Rag-1	1.502	100.	99.1	97.7
MR-481	1.613	7.2	7.2~~81~~	8.7
AB-15	1.605	98.7	97.1	100.

NB - Neither MR-481 nor AB-15 were agglutinated in 70% EtOH, while RAG-1 was.

Conclusions - AB-15 are as adherent as RAG-1 following growth on brain heart, despite their *in*agglutinability by both adsorbed and non-adsorbed ab (and by ethanol), and their lack of observable thin filaments in the EM.

In other words, the thin filaments which are responsible for agglutination of RAG-1, are not the whole answer.

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Fig. 1: Adherence of *RAG-1* and mutant MR-481 to hydrocarbon. Cells were inoculated 1:1000 into brain heart infusion broth ~~ang~~ ao and grown to stationary phase ~~at 30°c~~ (18h) at 30°c with shaking. Cells were then washed twice and resuspended in PUM ~~to an int initial turbidity of 1.45-1.5 (A400)~~. ~~Following~~

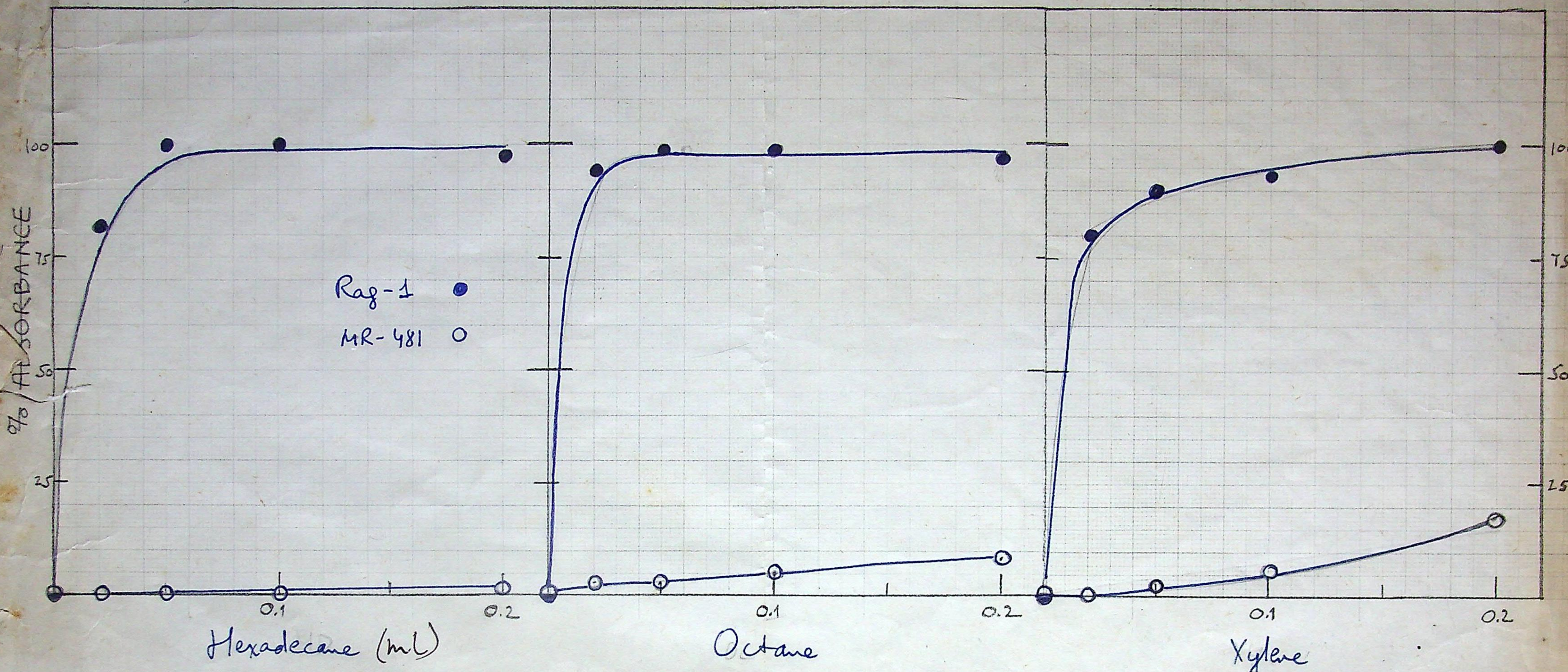
The assay for
adherence to hydrocarbon
s been described(6)
nd is reported
here in brief:

To 1.2 ml of a turbid cell suspension (1.45-1.50, A 400), various volumes of ~~the~~ test hydrocarbons were added and the mixtures vortexed under controlled conditions for 120 sec. Following phase separation the turbidity of the lower aqueous phase was recorded ~~and~~. and compared with that original bac. suspension. Results are presented as ~~percentage~~ % absorbance as a function of hydrocarbon volume added.

Fig. 2 Growth of *RAG-1* and mutant MR-481 ~~to~~ on hexadecane under conditions of limited ~~da~~ dispersion of the ~~aqueous~~ hydrocarbon phase.

Adherence of RAG-1 and Mutant MR-481 to Hydrocarbon.

22/7/80
and
29/10/80



Proc: Cells were grown O.N. (18h) on brain heart infusion broth, harvested and washed twice in PUM.
Adherence assayed as usual.
Initial O.D.$_{400}$: Rag-1 - 1.489
MR-481 - 1.463

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26/5 PRE BOOST
28/6 POST BOOST
1/7 WRITE UP

Preparation of antibodies against whole cells of RAG-1 and MR-481 grown to stationary phase on brain heart infusion

Bacteria were grown at 30°c to stat. phase on BHI, (24/4 batch) washed twice in water and ~~resusp~~ resuspended in PBS (pH 7.1, 20 mM P_i) to concentrations as indicated. Four rabbits were inoculated as follows; intravenously, no adjuvant.

Rabbit	Cells	Conc.
"כהה"	RAG-1	3×10^8/ml
"לבנה"	"	10^9/ml
"בהירה"	MR-481	3×10^8/ml
"שחורה"	"	10^9/ml

Pre-immune serum was collected prior to the 1st inoculation. Injections of 1ml were carried out as follows: 12/5, 15/5, 18/5. Rabbits were bled on 21/5 (pre-boost) and anti-sera tested for agglutination. On 10/6, a 1ml booster was given to each rabbit. Rabbits were bled again and sera tested for agglutination (post boost).

Agglutination: Washed (RAG-1 and MR-481) cells, grown as above (24/4 batch) were resuspended in PBS to initial $A_{400} \sim 1.4$. ~~Antiserum~~ Serum was added (5 µl for pre-boost test; 3 µl for post-boost test) and agglutination was followed turbidimetrically. Each serum was tested with both

Rag-1 and MR-481 cells.

Results- The results may be summarized in the agglutination table below, i.e; ∝ RAG-1 serum ~~pre~~ precipitates only RAG-1 cells; ∝ MR-481 serum does not precipitate either RAG-1 or MR-481 serum.

Antiserum Cells	∝ RAG-1	∝ MR-481
RAG-1	+	−
MR-481	−	−

Agglutination of RAG-1 cells by ∝ RAG-1 serum is shown in Fig. 1 (pre-boost, 5 μl antiserum) and Fig. 2 (post-boost, 3 μl antiserum). The $t_{1/2}$ for the 8 agglutinations are (in minutes):

	PRE-BOOST	POST-BOOST
"לבנה" ∝ RAG-1	240	135
"כהה" ∝ RAG-1	200	115

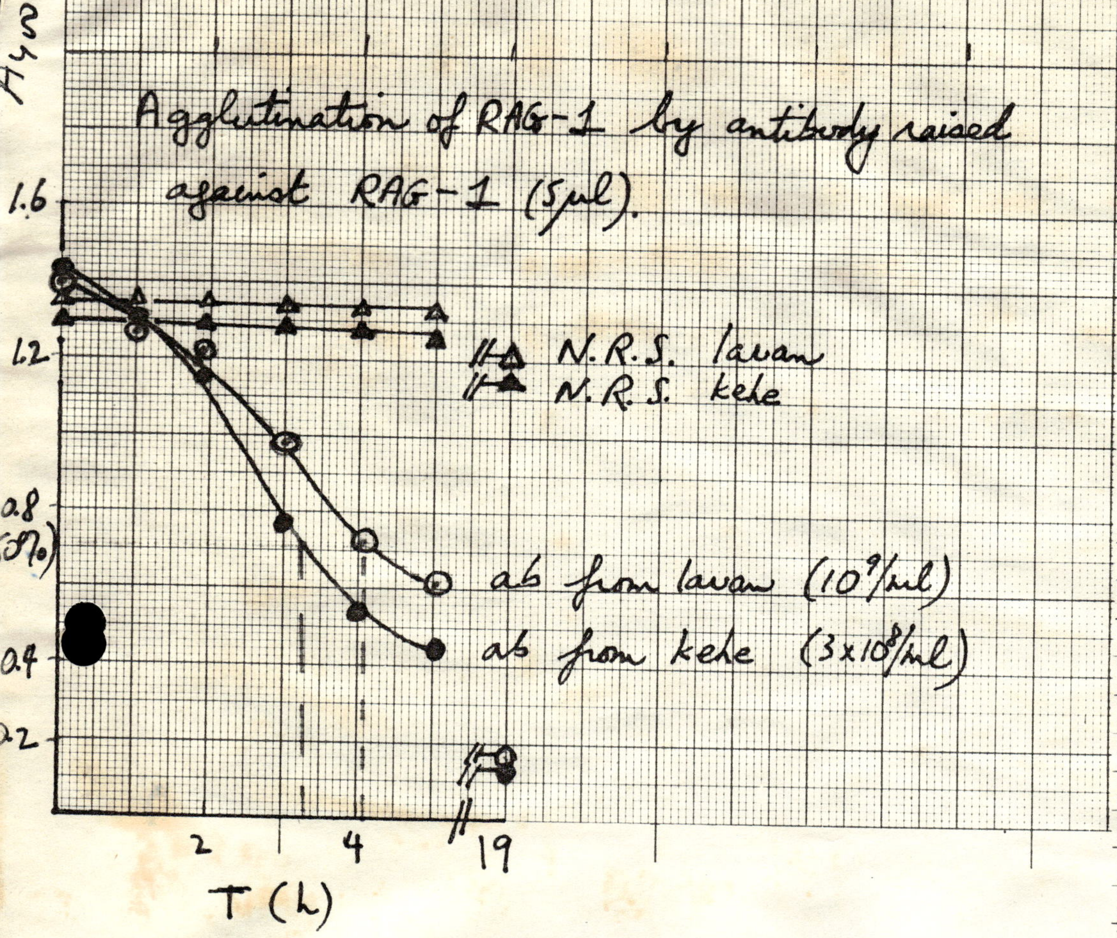
Results show that the (agglutination) titer has been significantly increased by the booster.

Conclusions-

1. MR-481 cells lack the antigen(s) responsible for the agglutinability of RAG-1 cells.
2. Antibodies vs. the agglutinating antigen are present in higher conc in the post boost, as opposed to the pre-boost anti-serum.

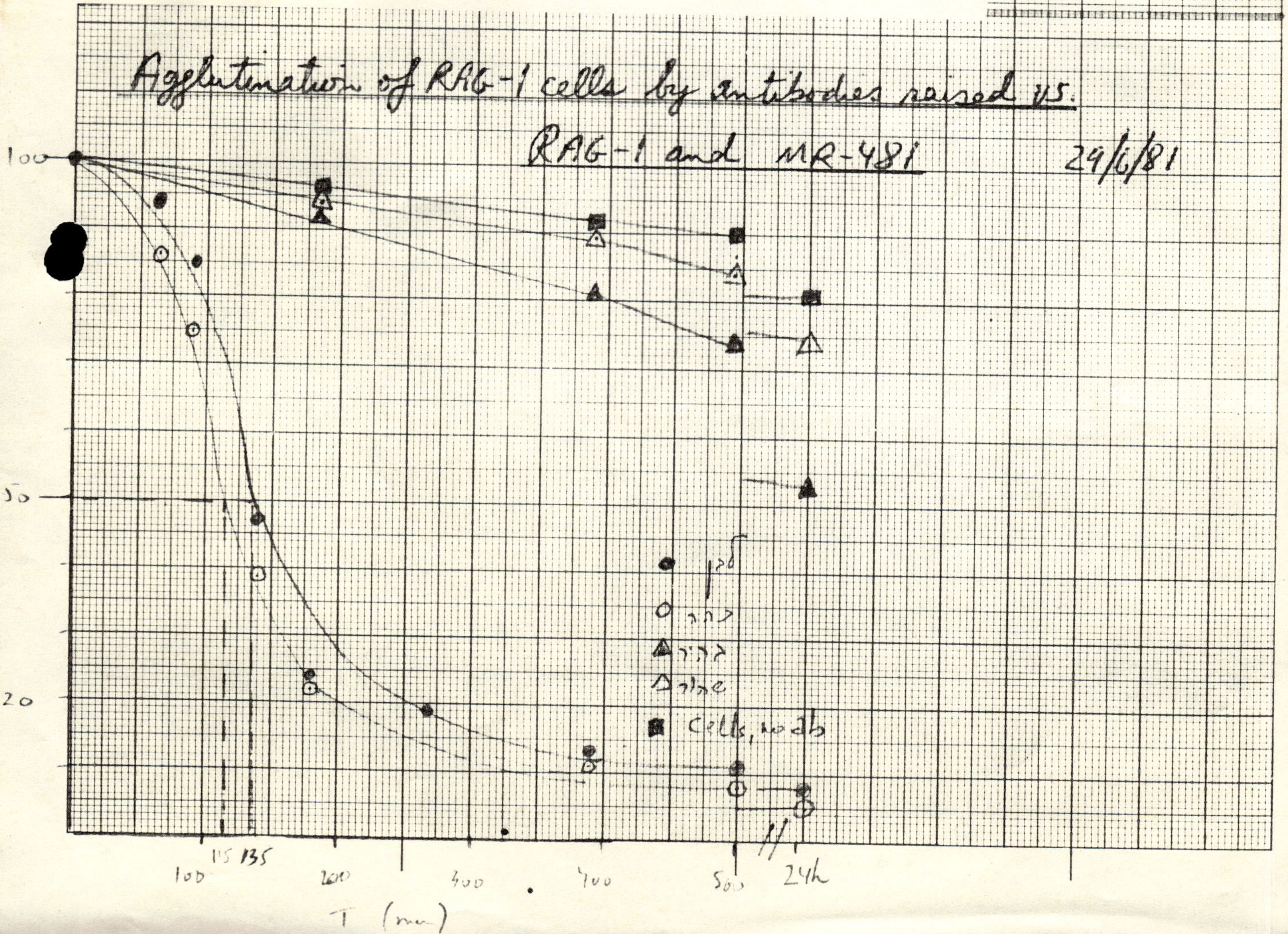
's to HD.

Agglutination of RAG-1 by antibody raised against RAG-1 (5µl).

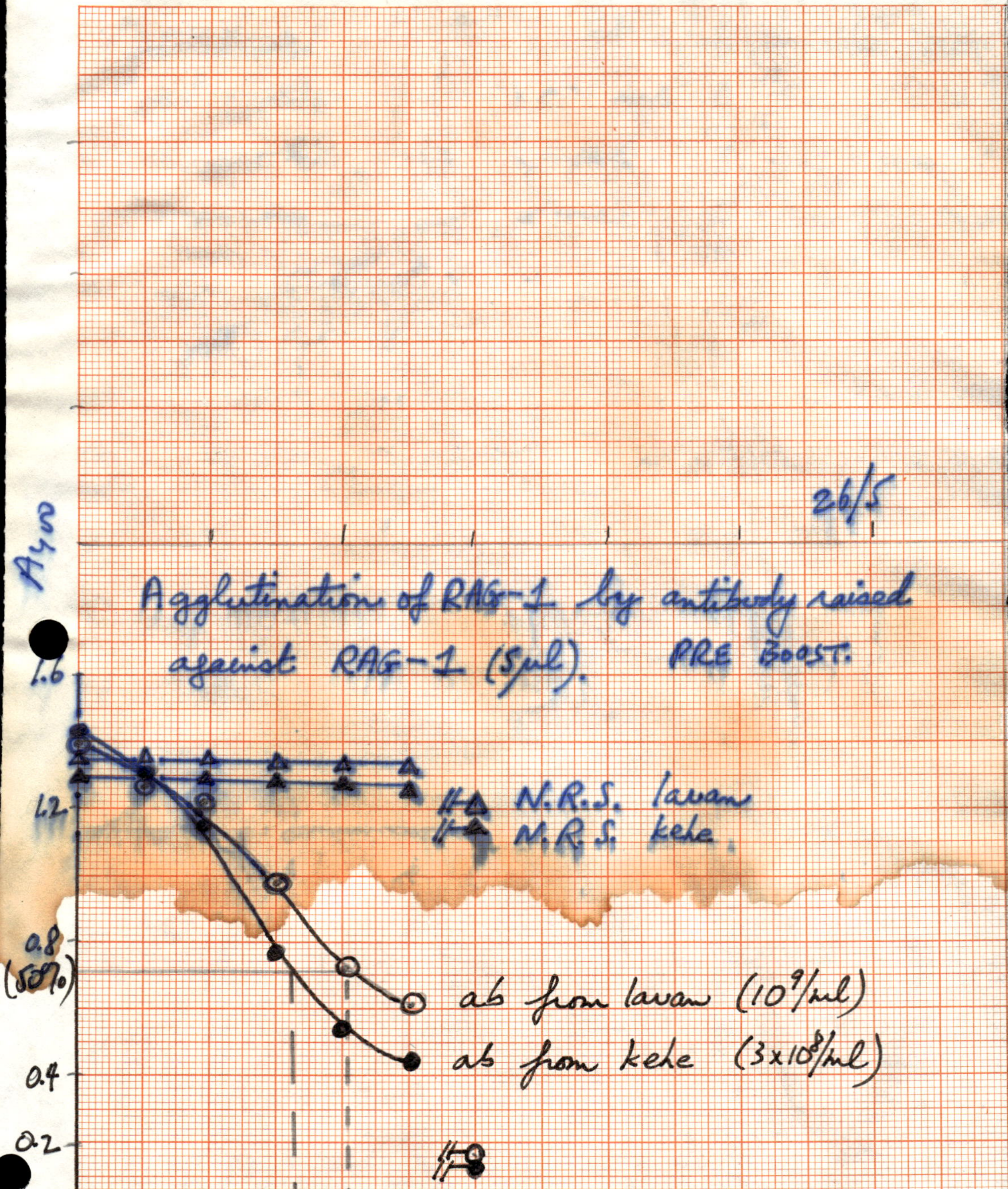


Agglutination of RAG-1 cells by antibodies raised vs. RAG-1 and MR-481

29/6/81



26/5/81.

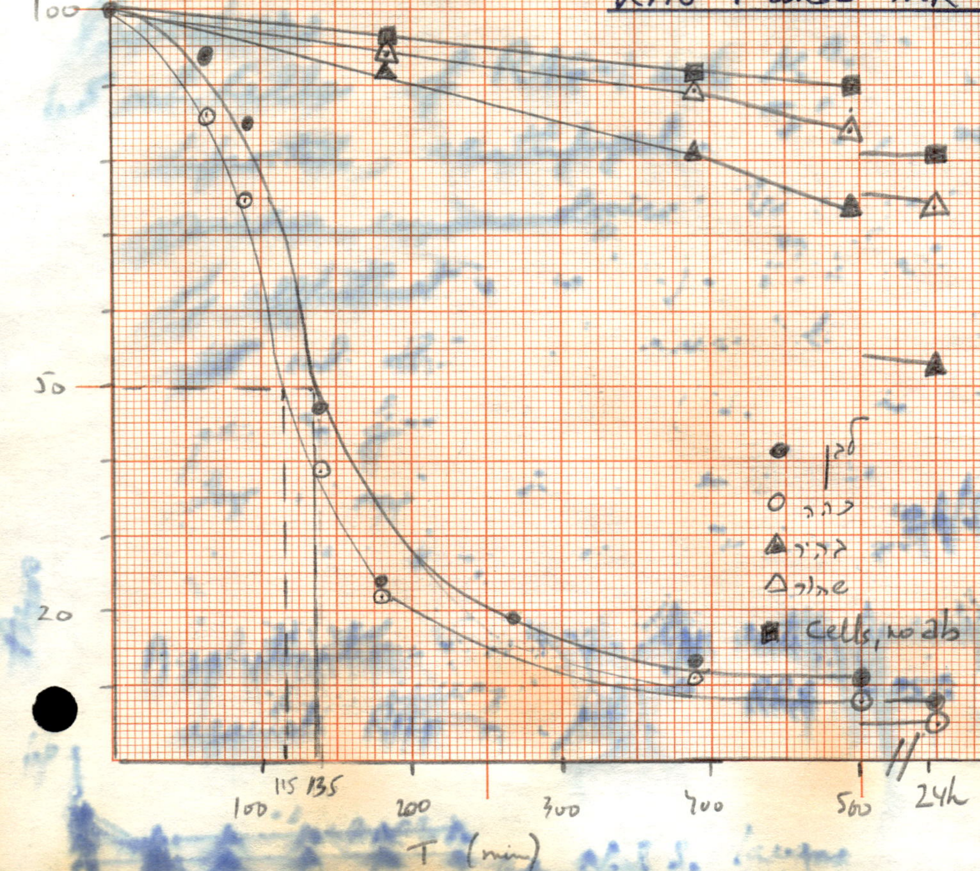


26/5

Agglutination of RAG-1 by antibody raised against RAG-1 (5µl). PRE BOOST.

Agglutination of RAG-1 cells by antibodies raised vs. RAG-1 and MR-481

29/6/81



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Sonication Experiment

17-21/5/81

Procedure:

1.5 ml. Cells of RAG-1 and MR-481 (batch: 24/4) were defrosted, centrifuged. The sups were taken for various immunological tests (ouchterlony, competition of agglutination, c.i.e.). Pellets were resuspended in 1.5 ml H_2O and sonicated at 0°C with the small probe for 30 sec (15"x2) at 70 Watts, spun down for 10 min. in eppendorf centrifuge. Rag-1 cells resuspended in PUM buffer and tested for adherence to hexadecane. Microscopic observation of the sonicate did not reveal broken cells + debris. Cells looked fine.

DROP IN A_{400}?

Adherence:

HD (μl)	Rag-1 control		Rag-1 sonicated	
	A_{400}	% adh	A_{400}	% adh
0	1.433	—	1.320	—
20	0.363	75	0.343	74
500	0.202	86	0.205	84
100	0.000	100.	0.134	90
200	0.000	100.	0.014	98.9

c.i.e.: The sonicate of RAG-1 cells contained 3 distinct bands which showed up with both preadsorbed and absorbed antibody; these 3 bands are missing from the MR-481 sonicate. The bands appear to be antigenically dissimilar.

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9/6/81

Preliminary comparison of adherence of Rag-1, MR-481, AB-15 and 92.

Growth - Cells were grown in

A. PUM + 2% EtOH + 50 μg/ml lysine - 48h - 30° (?).
B. BHI - 24h - 30° (?).

Adherence - Cells were washed resuspended in PUM and adherence to .100 and .200 ml Hexadecane tested as usual.

Agglutination - To 0.7 ml cells in PUM, 20 μl of 1:10 diluted MR-481-adsorbed Ed's antibody was added and following o.n. in fridge, ~~adsorbac~~ adsorbance compared.

Results -

1. Growth (K.U.)

15AB	EtOH	~~820~~	164
92	EthoH	~~960~~	192
MR 481	EtOH	~~2400~~	480
Rag-1	EtOH	~~2420~~	485
- - - -	- - -	- - -	
92	B.H.I.	~~1365~~	270
15	B.H.I.	~~1040~~	210

2. Adherence.

Strain	Growth on	Initial O.D.	% Adherence 0.1 ml	0.2 ml.
15AB	ethanol	1.469	8.7	13.4
92	"	1.357	13.6	36.0
MR 481 *	"	1357	8.0	13.2
RAG-1	"	1.532	40.0	51.4
92	B.H.I.	1.495	100.	100.
15	"	1.515	~~87~~ 86.7	94.6

* complete coalescence following mixing, both samples

3. Agglutination

Strain	Grown on	Initial		After o.n.		%
		+Ab	-Ab	+Ab	-Ab	Agglutinati nation
15	ethanol	1.390 ~~1.294~~ ~~1.390~~	1.427 ~~1.260~~ ~~1.427~~	1.361	1.376	0.4
92	"	1.273	1.322	0.151	1.171	91
481	"	1.287	1.318	1.105	1.091	0
Rag	"	1.432	1.502	0.178	1.254	86
92	BHI	1.371	1.399	0.074	1.212	94
15	"	1.260	1.294	~~0.754~~ 0.986	~~0.986~~ 0.754	0

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Sonicates of RAG-1 and MR-481   3/6- 10/6/80

1. Crossed i.e. — C.I.E. was run on the 2' and 6' sonicates of RAG-1 vs. and MR-481 against non-adsorbed and adsorbed (both 481 and 15) Ed's antibody. Rag-1 sonicate gave two or three bands which are missing in the MR-481 sonicate.

2. Inhibition of agglutination of RAG-1 cells with adsorbed (~~w~~with MR-481) antibody by sonicates—

Five and twenty µl of various sonicates were tested for their ability to inhibit RAG-1 (24/4 batch) ~~with~~ agglutination by antibody adsorbed with MR 481. To each cuvette, antibody and inhibitor (and H_2O to 200µl) were incubated overnight at 4° before cells were added. ~~A~~ From 0 to 8.5 hours, cuvettes were kept at room temperature, from 8.5 to 29 h. in fridge.

RAG-1 sonicates inhibited agglutination by up to 82%; MR-481 up to 30%. (see accomanying report)

Little difference (and little inhibition) was found for dialyzed spent broths of RAG-1 and MR-481 grown on brain heart infusion.

3. Dialysis and Centifugation of 6' sonicates -

The 6' sonicates of RAG-1 and MR-481 were extensively dialyzed against water. At this stage the sonicates appeared opalescent and many small particles could be seen. The sonicates were then spun for 60 min. in the ultracentrifuge (30,000 rpm, L-65 head). A pellet and clear (yellowish tinge) supernatant was obtained. ~~The volume at this stage of the sup was brought up from 3.5 ml up to and~~ The pellet was dispersed in 3.5 ml H_2O.

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Shearing of RAG-1 and MR-481

23-27/4/81

1. Cells ~~were~~ were grown to stationary phase on brain heart infusion @ 30°; washed twice and resuspended in 40 ml $H_2O \times 1$. One liter gave the following yields:

Rag-1:	[illegible] 0.46	g (dry wt)
MR-481:	0.40	g (dry wt).

20 ml. of each strain ~~were~~ were sheared three times at room temperature. Cells were spun down and resuspended in 10 ml $H_2O \times 1$; from this cells were diluted into PUM and adherence to 200 μl HD assayed:

2.

	RAG-1	MR-481
	% adh.	% adh.
control	100	1
sheared	99.2	2

A viscous supernatant was obtained which was dialyzed 72 h. in the cold against H_2O.

After dialysis the following volumes sup. were obtained:

RAG-1: 5.4 ml
MR-481: 7.9 ml.

The following volumes (included were removed as follows).

0.2 ml for emuls. assay
0.1 ml for Elisa
0.2 ml. for absorbance 280/260

Absorbance (1:30 diln.)

	RAG-1 Sup	MR-481 Sup
280	0.600	0.488
260	0.874	0.719

[illegible] MAE 1381 [illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible] 1.4

MAE 1381 25 mL

[illegible]

[illegible] 2.13

[illegible]

which corresponds to:

			% nucleic acid
RAG-1:	8.0 mg/ml x 5.4 ml =	43.2 mg	12%
MR-481:	6.3 mg/ml x 7.9 ml =	49.2 mg	12%

In other words, if 230 mg Rag-1 cells went to shearing, then the initial protein corresponds to ~ 115 mg and thus 43.2 mg in the dialyzed sup. corresponds to 37% of the original protein.

Emulsifying activity: 0.2 ml sup gave no emulsifying activity in either RAG-1 or MR-481 but little white pptt. formed in the aqueous phase.*

Movement on filter paper: RAG-1 did not move either before or after shearing. However, MR-481 moved before shearing and moved even better following shearing.

Elisa –

Elisa gave the following Emulsan equivalents:

	SAMPLE	TOTAL
RAG-1:	8.6 μg/ml x 5.4 ml =	46.4 μg
MR481:	6.5 μg/ml x 7.9 ml =	51.2 μg.

Dry weight after shearing:

			%
RAG-1:	13.9 mg/ml x 10 ml =	139 mg	60
MR-481:	15.6 mg/ml x 10 ml =	156 mg	78

* After freeze thawing, emulsifying activity repeated w/0.5 ml. from 9. No activity, white stuff only in MR-481 flasks, probably ~~re~~ related to freeze-thaw fluff.

3. The sups of both RAG-1 and MR-481 were diluted to 9 ml and frozen. Upon thawing a fluffy precipitate was formed. This pptt. was centrifuged and the sup was again checked for protein:

	RAG-1	MR-481
Dilution	1:6	1:10
A 280	0.247	0.402
A 260	0.390	0.647
Tot. Protein	4.7 mg	11.8 mg
% N.A.	14	15
% previous prot.	11%	24%

i.e. most of the protein was removed by freezing and thawing.

Note: The stuff pptting from RAG-1 was more difficult to get back into the aqueous phase.

4. Conclusions -

A. Shearing probably lyzed a high proportion of the cells and had little or no effect on adherence of RAG-1. Similarly the process did not enhance the non-adherence of MR-481.

B. The supt. contained small but finite amt. of Emulsan c.r.m. material.

C. Most of the protein could be precipitated by freezing and thawing of the dialyzed sup.

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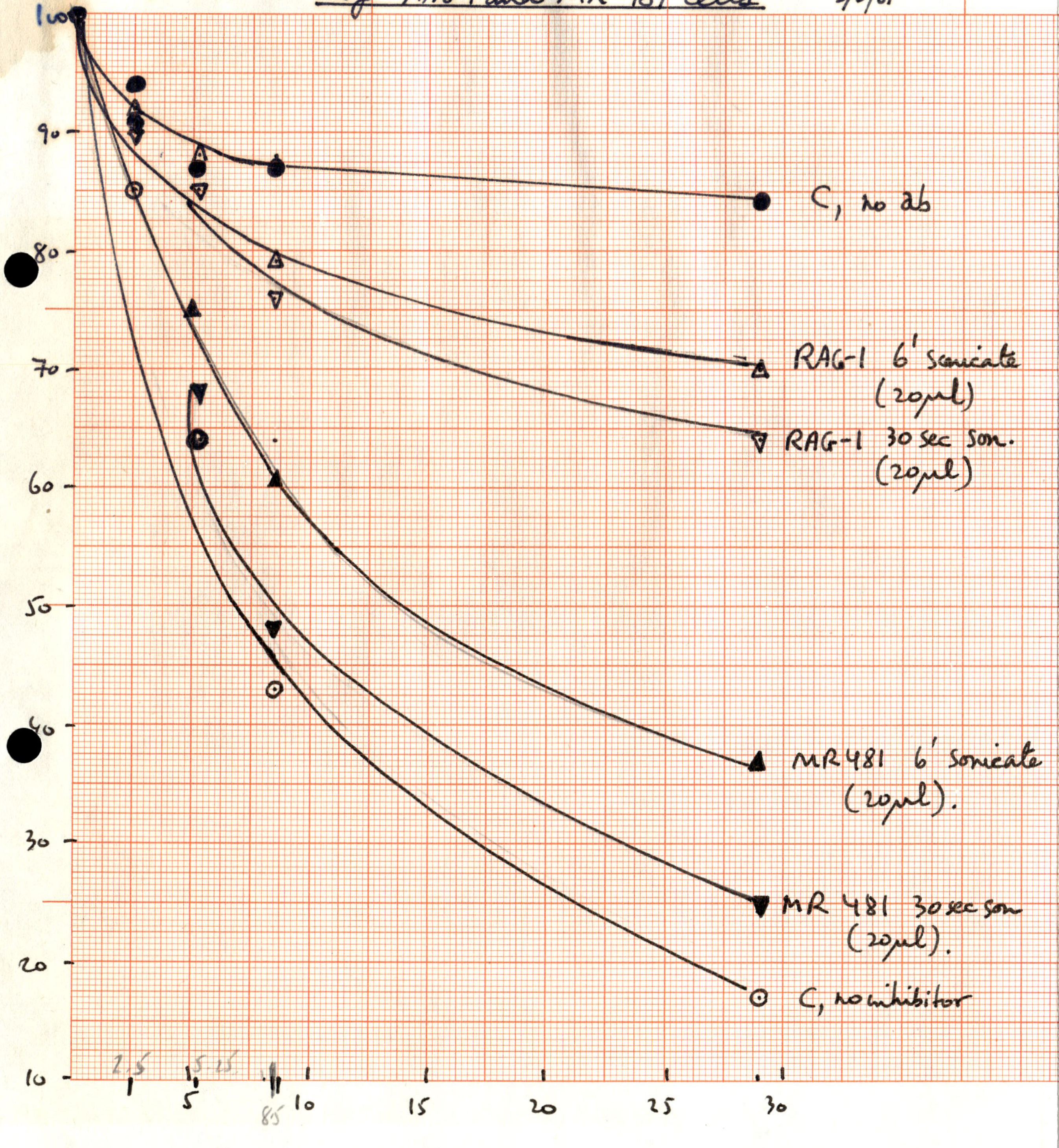
Inhibition of Agglutination by sonicates 3/6/81

Sonicates, antibody (Ed's antibody adsorbed with MR-481, diluted 1:10) and water to 200 µl were preincubated at 4°C overnight. Rag-1 cells (24/4 batch), washed and resuspended in PBS were added to a final volume of 700µl and turbidity (A_{400}) was measured as a ~~fcn~~ function of time.

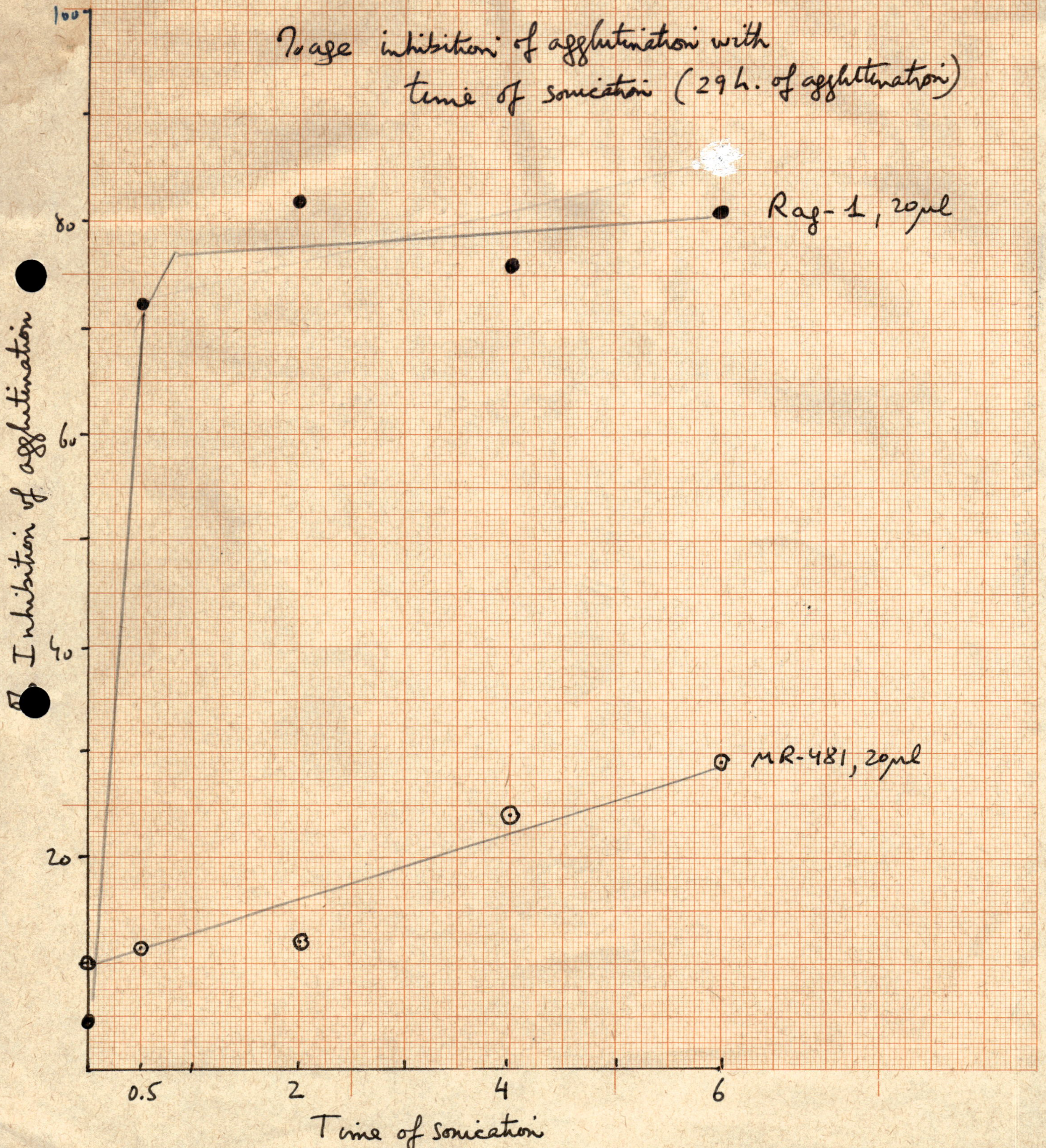
Sample no	Sample vol. µl	1:10 Ab vol µl	H_2O vol µl	RAG-1 in PBS	Total Vol	Sample
1	20	10	180	500	710	RAG sonicate T_0
2	5	↓	195	↓	↓	" $T_{1/2}$
3	20		180			"
4	5		195			" T_2
5	20		180			"
6	5		195			" T_4
7	20		180			"
8	5		195			" T_6
9	20		180			"
10	20		180			MR 481 sonicate T_0
11	20		180			" $T_{1/2}$
12	20		180			" T_2
13	20		180			" T_4
14	5		195			" T_6
15	20		180			"
16	200		–			BHI broth } dialyzed vs. H_2O
17	200		–			" RAG-1 sup } dialyzed vs. H_2O
18	200		–			" MR481-sup } dialyzed vs. H_2O
19	–		210			} control, no additions
20	–	↓	210	↓	↓	} control, no additions
21	–	–	~~21~~ –	700	700	Control, no ab.

Agglutination of RAG-1 in presence of sonicates of RAG-1 and MR-481 cells

3/6/81



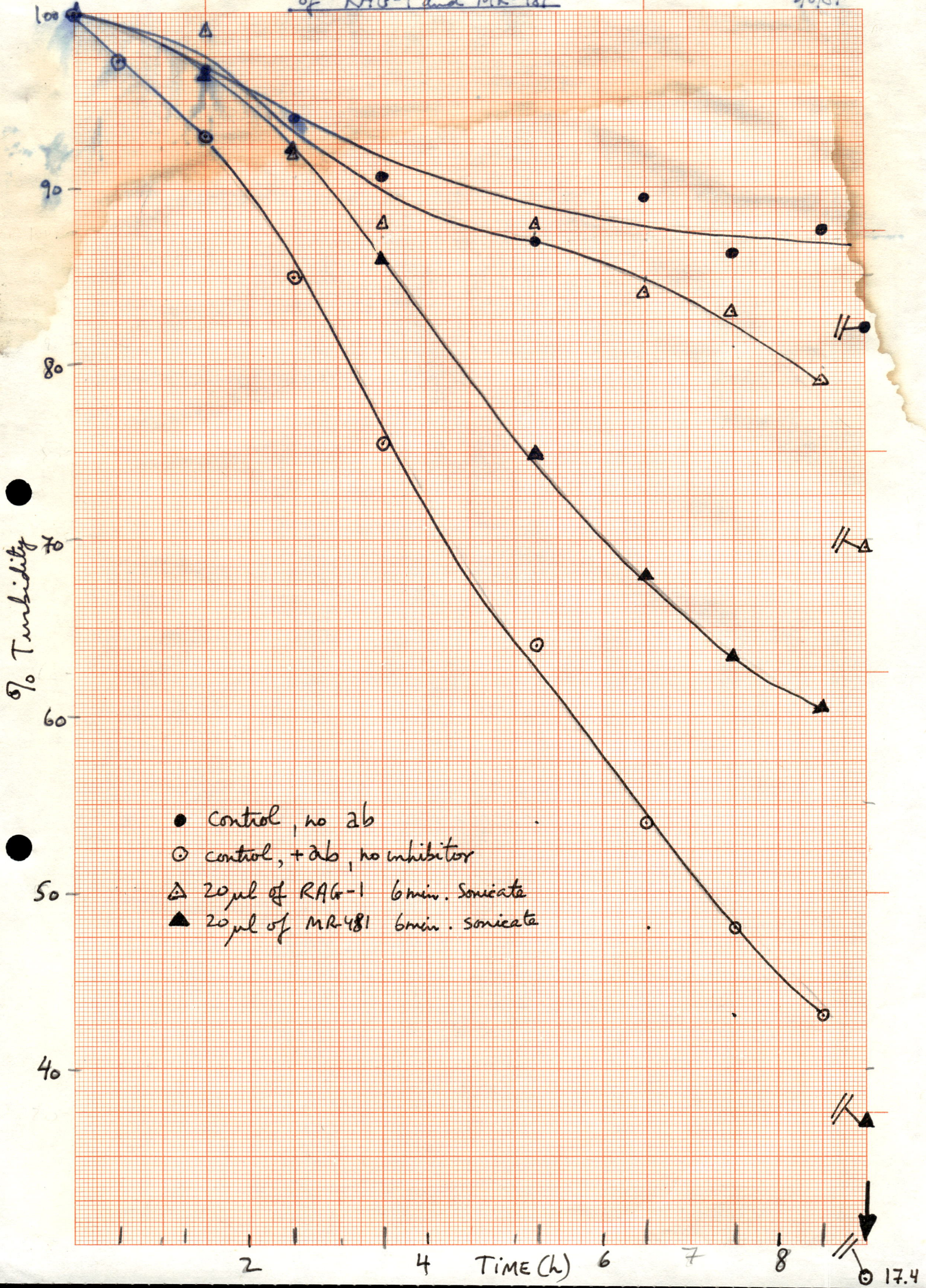
of time of sonication, following 29h.

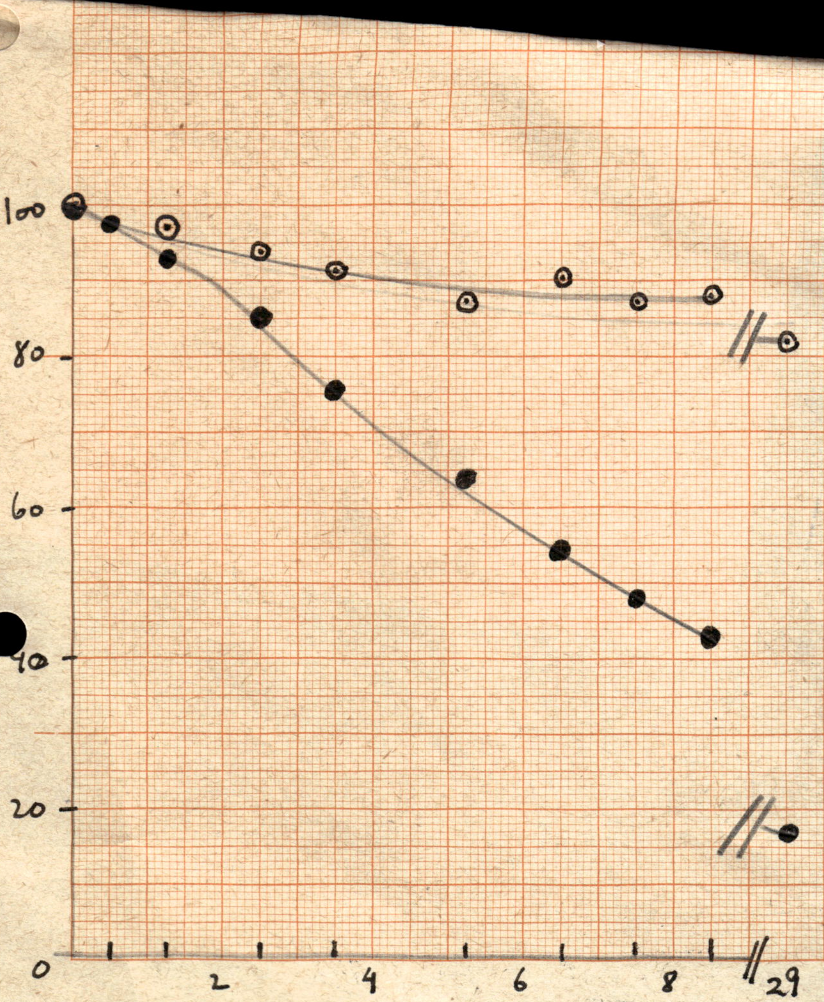


%age inhibition of agglutination with time of sonication (29h. of agglutination)

Agglutination of RAG-1 in presence of sonicates from of RAG-1 and MR-481

3/6/81




100
80
60
40
20
0
2
4
6
8
29

Sample No	Initial O.D. A_{400}	Time (H)								
		0.5	1.25	2.5	3.5	5.25	6.5	7.5	8.5	// 29
1	1201	96.8	91.0	84.5	76.4	61.8	50.7	44.5	41.2	20.3
2	1231	96.7	91.9	85.5	80.5	79.5	75.1	70.4	69.6	47.8
3	1231	98.5	93.7	89.9	87.7	84.9	82.2	74.4	76.1	63.9
4	1241	96.5	93.8	85.9	81.8	75.5	69.3	65.3	62.7	44.0
5	1255	97.0	96.0	90.8	87.2	87.0	84.8	80.9	79.7	70.4
6	1213	98.7	94.5	89.9	81.9	80.0	78.4	73.6	71.3	51.9
7	1250	98.2	96.2	94.8	88.5	87.2	85.1	85.3	82.2	66.4
8	1256	95.5	93.4	86.5	82.5	79.5	75.1	70.6	70.1	48.3
9	1318	99.5	98.9	92.2	88.0	88.2	84.1	82.8	78.8	69.8
10	1191	98.6	90.6	85.9	78.2	68.4	62.0	57.7	49.4	23.8
11	1191	100.5	94.8	90.9	80.6	67.8	50.5 ~~61.0~~	53.1	47.6	24.9
12	1254	96.5	92.9	88.0	79.2	67.5	[illegible]5.3	56.9	52.2	25.3
13	1275	99.5	97.3	87.4	85.1	71.1	62.7	60.0	54.2	31.8
14	1249	100.0	95.8	90.3	85.3	76.0	70.5	66.2	61.5	38.2
15	1301	98.8	96.5	92.5	86.1	74.9	68.0	63.6	60.6	36.8
16	1322	97.7	90.9	76.7	66.3	47.9	38.7	33.0	30.1	13.2
17	1302	97.2	95.0	88.7	80.5	61.3	51.6	44.4	40.6	23.7
18	1295	98.9	94.6	89.6	80.7	56.1	46.9	39.8	36.1	21.7
19	1215	97.9	93.5	84.9	75.2	63.0	51.9	44.4	39.5	17.3
20	1197	97.1	92.5 ~~84.8~~	84.8 ~~75.9~~	75.9 ~~65.2~~	65.1 ~~55.8~~	55.8	52.0	46.4	17.5
21	1580	—	96.7	93.9	90.7	87.0	89.6	86.6	87.6	818

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see also 7-8/4/81

Acid Treatment

24/4/ – 1/5/81

Procedure

Cells from the batch of 23/4 were used, following their resuspension in 10ml.

Thus dry weights:

Rag-1 : 460 mg/20ml → 23 mg/ml → 115 mg/5ml

MR-481: 400 mg/ml → 20 mg/ml → 100 mg/5ml.

Cells were subjected to the following treatments:

A) none
B) 0.05 N HCl at 0° for 60'
C) " 100° for 5'
D) " 100° for 30'

Following treatment, cells were spun down. pH of sups was between 1.6 – 1.7. The sup's obtained were not viscous. Sups were dialyzed 48 h against H_2O until pH = 6.

Adherence (to 200 µl +500 µl hexadecane).

	Rag-1			MR-481		
Sample	C	200µl	500µl	C	200µl	500µl
A	1.333	100%	—	1.208	1%	—
B	1.841	19%*	31%	1.553	0%	0%
C	1.701	8%	63%	1.652	21%	76%
D	1.798	14%	52%	1.758	10%	48%

* cells came onto the HD after 30sec but then came back off.

The resuspended RAG-1 and MR-481 cells ("D"-treated) were not pptted by 90% EtOH (neither were MR-481)

Three ml. of cells (i.e. 69 mg Rag-1 and 60 mg MR-481), treated w/ 0.05N HCl for 30min. @100°C were spun down. The sups, which ~~was~~ were not viscous, were dialyzed against water until pH 6. The sups became viscous and had a yellow ppt, which upon mixing with hexadecane and vortexing, finely emulsified the hydrocarbon.

The sups were diluted to 9ml and tested for emulsifying activity, 280/260 absorbance, elisa

Absorbance - (no dilution)

	260	280	Prot/ml	Tot prot (mg)	% N.A.	חלבון/משקל יבש
Rag-1	1.392	1.104	0.65	5.9	6.6	8.6%
MR481	1.068	0.819	0.46	4.1	7.0	7.7%

Emulsifying activity (0.3cc) - NONE.

Elisa - NONE

Conclusions - This method is promising because although the conditions employed are harsh, adherence and flocculation properties of the wild type cells are both affected. Moreover, the pellet contains some kind of emulsifying activity.

The dialyzed sup became viscous and had a yellow precipitate. The sup was diluted to a volume and checked for:

0.2 ml → emulsifying activity

260/280 absorbance

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Eddie and Mel - 19/5/81

Competition of cell extracts for agglutination of RAG-1 by MR-481-adsorbed antibodies.

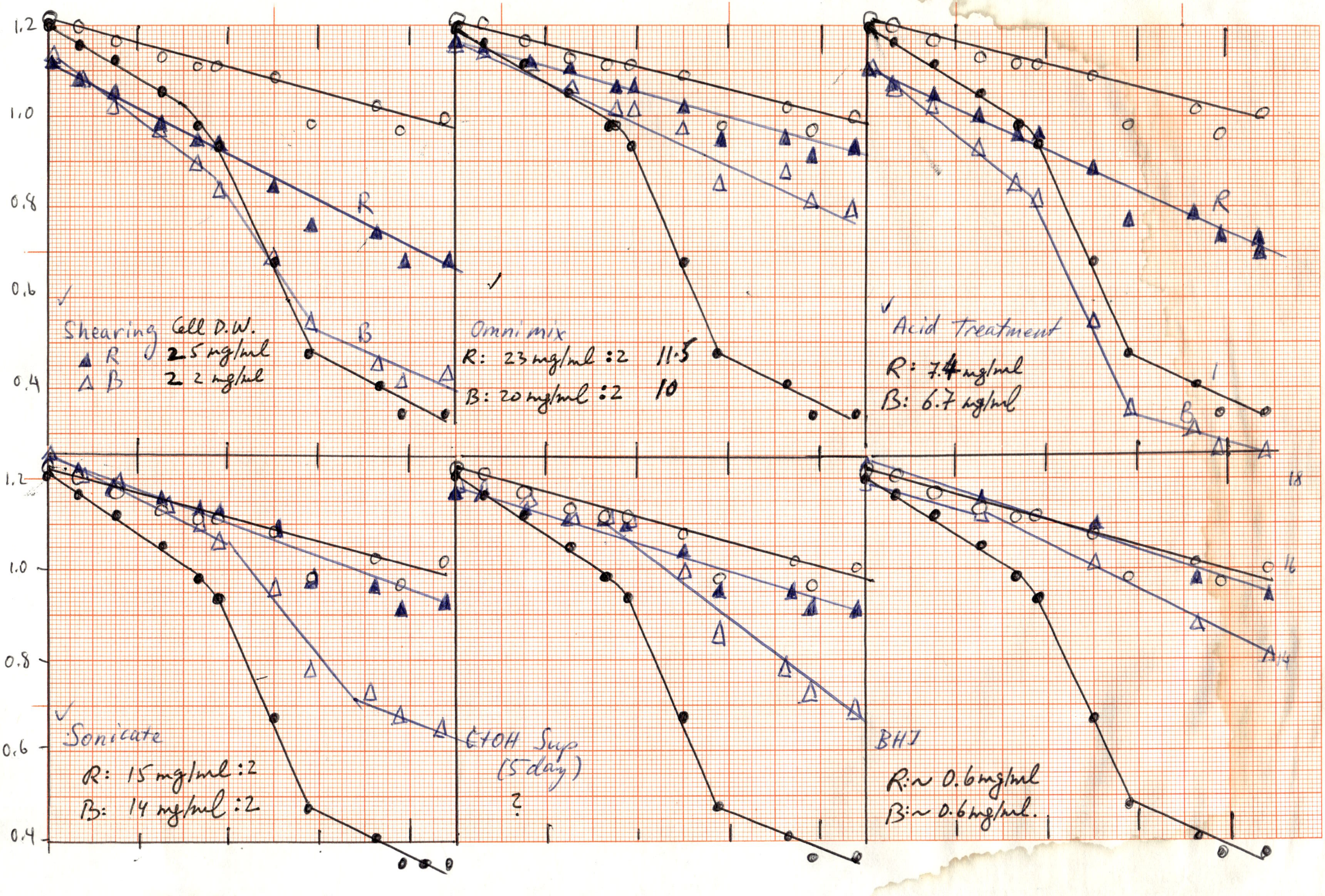
Method -

Ten µl of Ed's antibody, adsorbed by MR-481, was incubated with concentrated or diluted extracts as indicated overnight at ~~to~~ 4°C. Rag-1 cells (batch of 24/4) in PBS (pH7.1, 20mM P_i) were then added. Agglutination was measured as drop in A_{400} as a function of time at room temperature

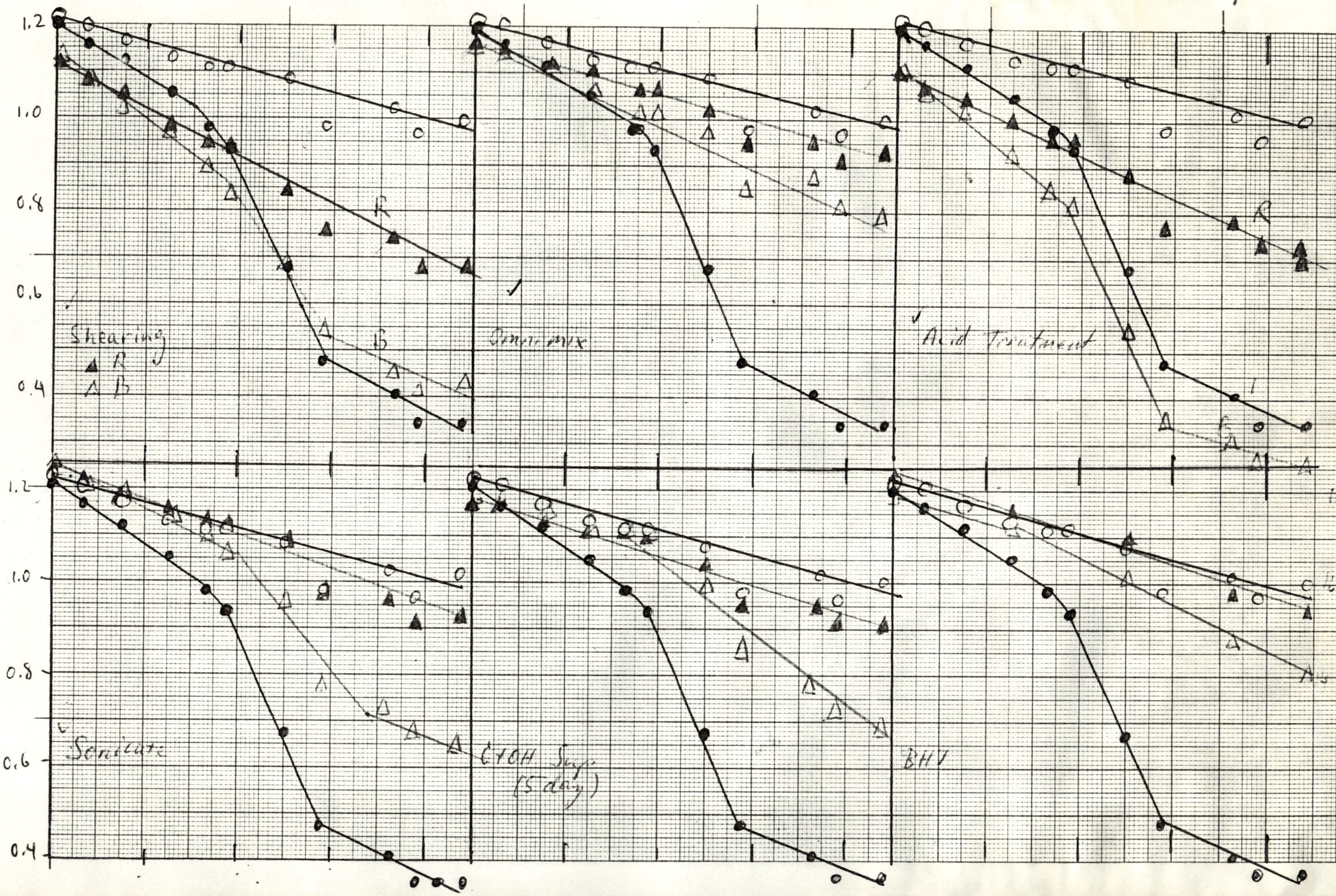
No	Extract Sample	Sample Volume	MR481-adsorbed Ed's Antibody (1:10 dilution)	H_2O*	Rag-1 in PBS.	Tot vol.
1	Shearing (Rag)	200µl	10µl	–	500µl	710
2	" (481)	200µl	↓	–	↓	↓
3	Omnimix (Rag)	100		100		
4	" (481)	100		100		
5	acid treatment (Rag)	200		–		
6	acid treat (481)	200		–		
7	Sonicate (Rag)	100		100		
8	" (481)	100		100		
9	EtOH 5day sup Rag	200		–		
10	" 481	200		–		
11	BrainHeart sup Rag	200		–		
12	" 481	200		–		
13	Control (no extract)	–		200		
14	Control (no antibody)	–	–	200		

* added w/antibody (before o.n. incubation).

19/5


1.2
1.0
0.8
0.6
0.4
R
B
✓
Shearing Cell D.W.
▲ R 2.5 mg/ml
△ B 2 mg/ml
✓
Omnimix
R: 23 mg/ml :2 11.5
B: 20 mg/ml :2 10
✓
Acid Treatment
R: 7.4 mg/ml
B: 6.7 mg/ml
1
B
18
16
14
1.2
1.0
0.8
0.6
0.4
✓
Sonicate
R: 15 mg/ml :2
B: 14 mg/ml :2
EtOH Sup
(5 day)
?
BHI
R:~ 0.6 mg/ml
B:~ 0.6 mg/ml.

19/5


1.2
1.0
0.8
0.6
0.4
Shearing
▲ R
△ B
R
B
Omnimix
Acid Treatment
R
B
1.2
1.0
0.8
0.6
0.4
Sonicate
C1OH Sup.
(5 day)
BHI

Results -

A_{400}

TIME (h.)

TIME (h)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.	1.113	1.117	1.171	1.168	1.104	1.105	1.236	1.275	1.159	1.184	1.812	1.773	1.205	1.217
0.67	1.078	1.077	1.148	1.141	1.073	1.065	1.207	1.195	1.143	1.185	1.795	1.759	1.169	1.195
1.5	1.051	1.024	1.119	1.116	1.049	1.019	1.168	1.185	1.136	1.165	1.777	1.731	1.122	1.167
2.5	0.985	0.970	1.098	1.043	0.993	0.929	1.155	1.137	1.115	1.115	1.762	1.710	1.050	1.131
3.3	0.942	0.890	1.054	1.017	0.965	0.847	1.127	1.105	1.100	1.103	1.730	1.679	0.981	1.111
3.8	0.929	0.829	1.066	1.020	0.948	0.817	1.112	1.068	1.088	1.082	1.721	1.666	0.938	1.112
4.9	0.840	0.678	1.028	0.971	0.886	0.546	1.072	.965	1.024	0.992	1.687	1.607	0.677	1.083
5.7	0.763	0.530	0.944	0.848	0.760	0.345	0.964	.787	0.929	0.841	1.594	1.495	0.477	0.983
7.3	0.737	0.455	0.952	0.846	0.784	0.303	0.962	.728	0.940	0.785	1.569	1.475	0.405	1.014
7.8	0.677	0.417	0.905	0.802	0.727	0.261	0.909	.679	0.909	0.733	1.543	1.424	0.340	0.965
8.8	0.678	0.436	0.937	0.798	0.732	0.257	0.935	.660	0.906	0.703	1.544	1.409	0.341	1.001

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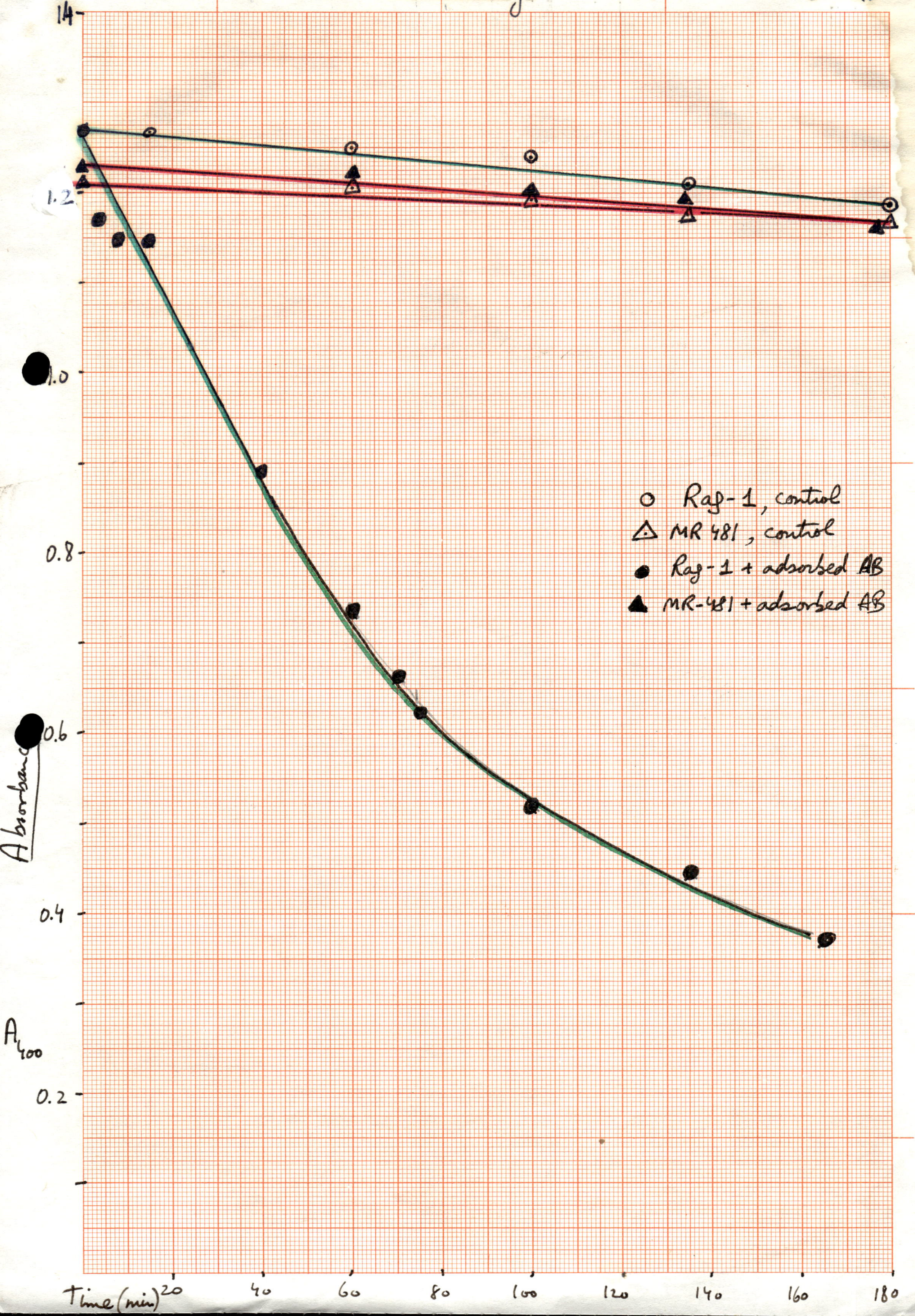
First Adsorption Expt   Ed and Mel   14/5/81

Five ml. of cells of MR-481 in H_2O (batch of 24/4) were spun down (the supt. was kept frozen) and resuspended in 2 ml PBS (pH 7.1, 20 mM Pi). Total Dry Wt. of cells was 72 mg. One ml. of Eddie's IgG antibodies, raised against RAG-1 cells growing on ethanol (March 1980) were added and agglutination was immediately observed. The mixture was incubated overnight in the cold, then spun down, yielding the sup.

Three µl of antibody were added to 1 ml. of RAG-1 and MR-481 cells (batch of 24/4) ~~resusp~~ resuspended in PUM to initial A_{400} of ~1.2. Loss of turbidity due to agglutination of cells was measured as a function of time

TIME (mins.)	RAG-1	RAG-1 + ab	MR-481	MR-481 + ab
0	1.272	1.272	1.216	1.231
4	–	1.172	–	–
8	1.269	1.149	1.214	1.229
15	1.266	1.144	1.212	1.227
40	1.269	.892	1.214	1.226
60	1.250	.735	1.209	1.222
70	–	.659	–	–
75	1.250	.616	1.208	1.215
100	1.236	.517	1.193	1.203
135	1.212	.446	1.175	1.188
165	–	.371	–	–
180	1.185	.364	1.167	1.163

Agglutination of MR-481 and RAG-1 by adsorbed antibody. 14/5/81 Mel+Ed.



14/5/81

Adsorption of antibodies by MR-481

Mel and Eddie

1. Adsorption -

To 1 ml. of IgG raised against RAG-1 grown on ethanol, were added 5 ml of MR-481 (72 mg dry wt), suspended in ~~H_2O~~

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Ed + Mel
18/5/81

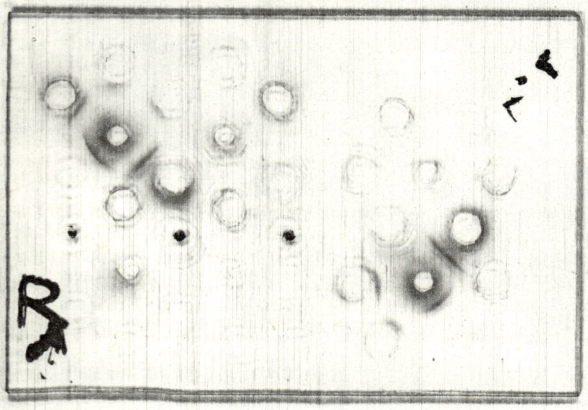
Ouchterlony

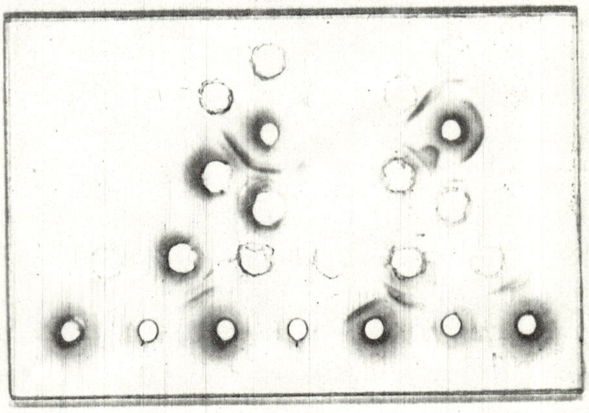
Conditions -

I - Preadsorbed antibody (Ed's) } 7 μl
II - Antibody adsorbed by MR 481. }

Extracts - 15 μl as indictated.

1. Final sup from shearing.
2. Omnimix sup
3. acid treatment sup
4. sup following thawing of frozen batch of 24/4
5. sonicate sup
6. spent brain heart infusion following growth to stationary phase.





Crossed Immunoelectrophoresis of RAG-1 and MR-481 Sonicates Summary

MEL AND ED

Approach:

1. Preparation of antiserum against intact cells of wild-type, i.e., anti-cell surface antiserum AS_{wt}

2. Adsorption of above antiserum onto mutant cells in order to remove antibodies common to wild-type and mutant ads (481). The antibodies left should be specific to surface antigens exposed on wild-type cells only.

3. Using the above two antisera, both wild-type and mutant sonicates were examined by crossed immunoelectrophoresis (CIE). Four CIE patterns were thus obtained:

	Sonicates	
Antiserum	Wild type	Mutant
AS_{wt}	A	B
ads 481	C	D

CIE PATTERN A:

Wild-type antiserum versus wild-type sonicate

General pattern of surface components on wild-type

CIE PATTERN B:

Wild-type antiserum versus mutant sonicate

General pattern of surface components on mutants

CIE PATTERN C:

Mutant-adsorbed antiserum versus wild-type sonicate
Pattern of surface components specific to wild-type and absent (or unexposed) on mutant surface.

CIE PATTERN D:

Mutant-adsorbed antiserum versus mutant sonicate

Extraneous bands represent incomplete adsorption.

Summary of Results (Mel & Ed)

1. About 10 components consistently appear in RAG-1 sonicates (CIE PATTERN A)
2. Using ads (481) antibodies, 3 components are present in wild-type RAG-1 sonicates, but are absent in mutant 481 sonicates.
3. A similar pattern is obtained with antiserum adsorbed onto mutant AB15 (agglutination-less), indicating a similarity between the surfaces of AB15 (ethanol grown) and MR481-B.
4. These three components are suspect (either alone or in concert) as cell-surface agglutinogen(s) and/or as factor(s) contributing to cell surface hydrophobicity.
5. Comparison of CIE patterns of wild-type and mutant sonicates at varying times (30 sec, 2 min, 6 min) of sonication, indicates that 2 min of sonication appears optimum.

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Growth data of 92 and 15 on hexadecane 8/11/81

Strains 92 and AB-15 were tested for growth on hexadecane following growth on:

(1) brain heart infusion (stat. phase, 20h.).
(repeated twice). 4/10, 2/11
(2) plates supplemented with hexadecane vapours 25/10

1. Brain heart infusion - 4/10. into PUM + 20µg/ml lysine + 0.5ml HD

HD		TIME (H). (KU-uncorrected)				
		0	20	49	72	118
Rg	92	5	15	27	95	228
	92	5	13	56	78	118
	92	5	13	45	108	173
AB	15	5	13	13	14	12
	15	5	13	7	10	9
	15	5	14	12	16	72

2. Acetate (0.2%).

		TIME (min.).										
		0	135	260	315	345	375	405	465	495	550	585
Rag	92	5	8	28	53	66	85	102	134	152	180	196
AB	15	5	5	5	5	5	9	18	46	71	106	129

		1260.
Rag	92	218
AB	15	218

2. Brain heart - 2/11/81 PUM+ 50 μg/ml lysine + 0.5 ml HD

HD	Time (hr).				
	0	23	42	72	140
Rag 92	5	5	20	30	48
Rag 92	5	5	42	35	68
AB 15	5	6	4	5	5
AB 15	5	6	6	8	–

0.2% Ac	Time (hr).					
	0	3	4.25	6.	7	16
Rag 92	5	13	23	57	70	98
AB 15	5	9	16	41	60	93

Control- PUM + 0.2% Ac, no lysine.

after 6 days: Rag-92: 3 K.U.
AB-15: 8 K.U.

3. Pregrown on $\frac{PUM}{2}$ plates + HD vapours + 100 μg/ml lysine 25/10
into PUM+ 50 μg lys + 0.5 ml HD

HD	Time (hr).					
	0	24	49	61	71	88
Rag 92	5	3	6	57	54	50
AB 15	5	6	4	6	3	5

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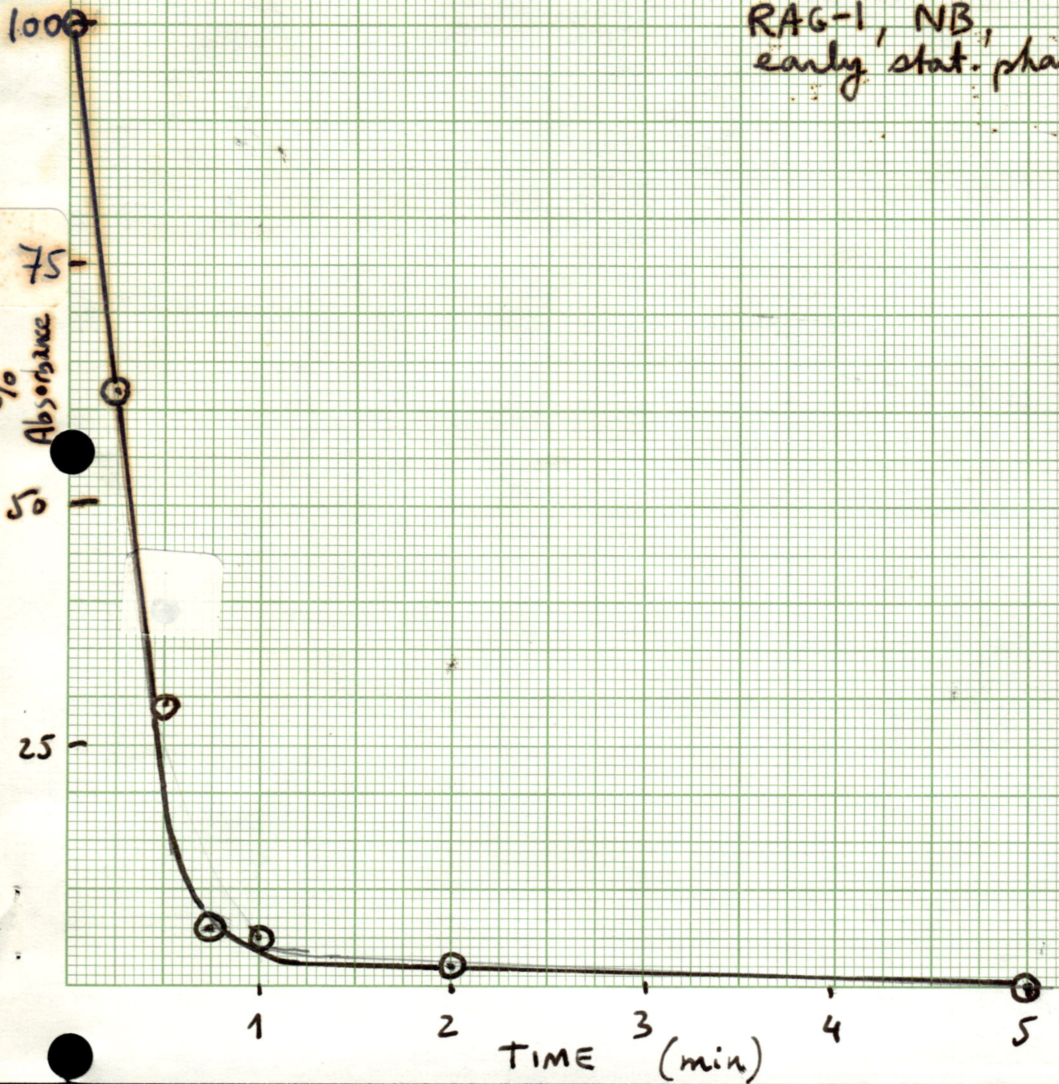
Fig. 4

18/11/81



Effect of time of vortexing on % absorbance.

RAG-1, NB,
early 'stat.' phase (5.5h)

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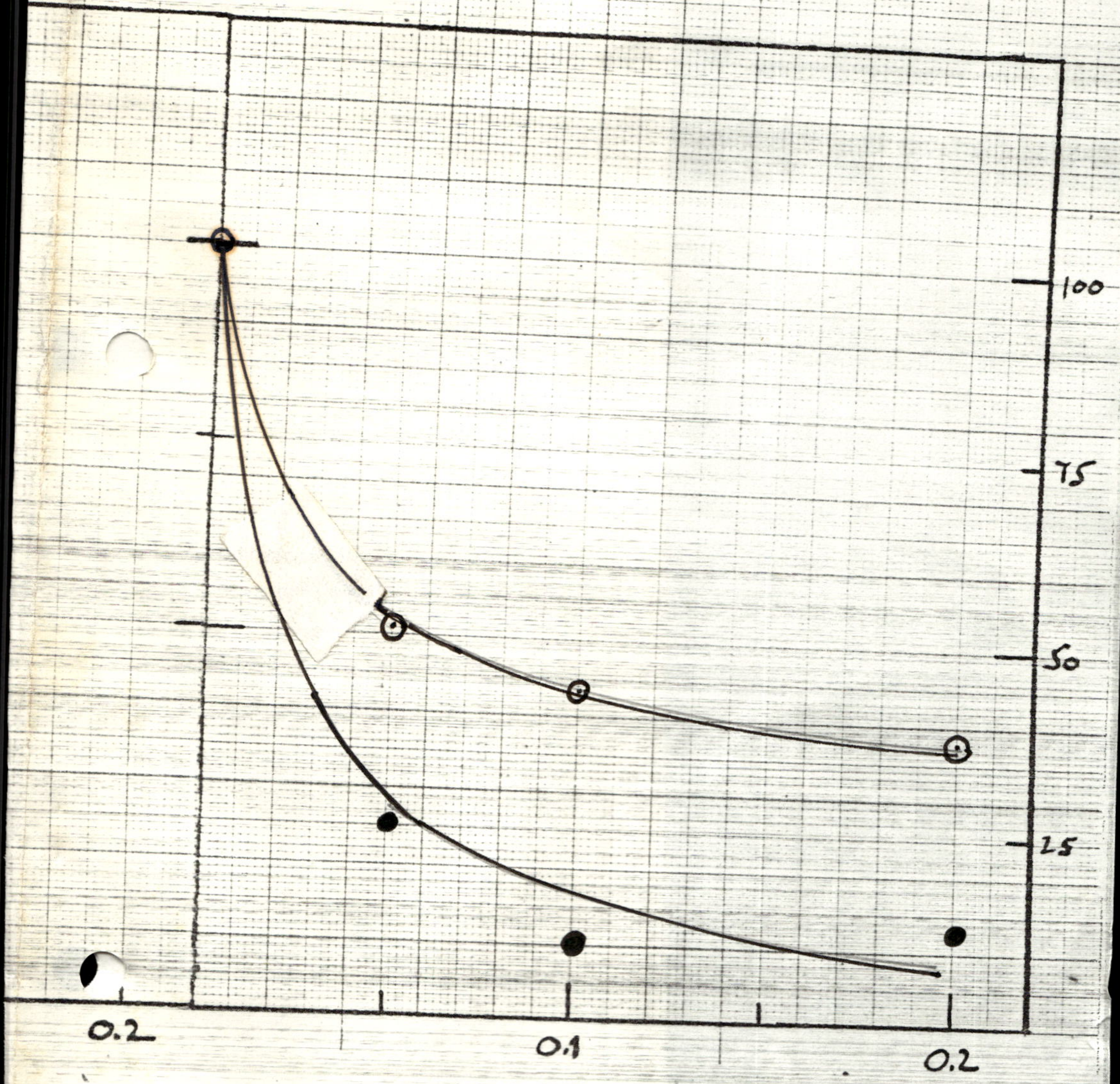
Fig. 20

13/9/81



T_0 ● ~~1.463~~ 1.435

T_5' ○ 1.463

p.28

3/9/81

0.1

יורם - מרץ 82'

שוטפים את התאים פעמיים ב PUM מבעים

Effect of enzyme on adherence of RAG-1 cells grown on EtOH vapours.

ל ע.ו 120

מחלקים ל מבחנות 0.2 מל

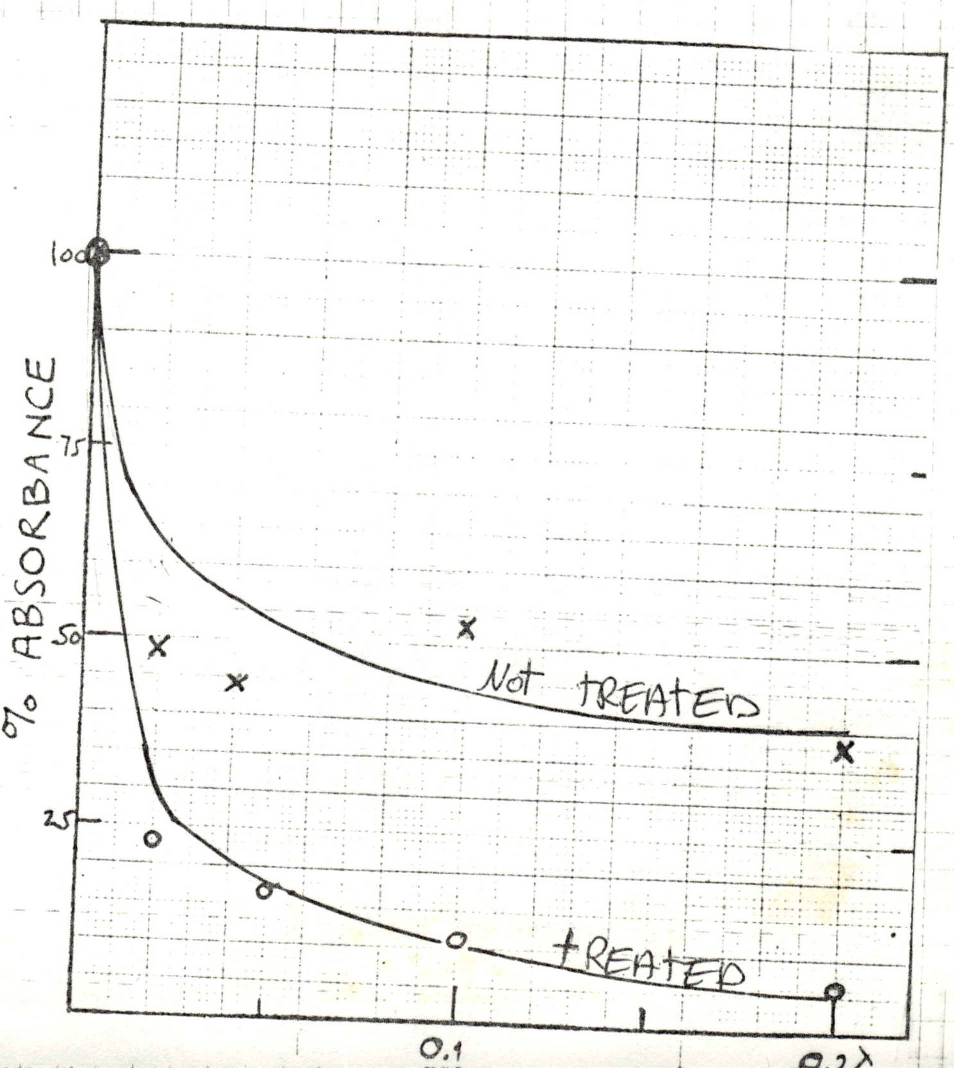
מוסיפים 20x 50x 100x ו 200x הקספקין

ורטקס 2 דקות. עד שניות הפסקה ז שניות

4 פעמים.

תוצאות

	מטופלים	%	לא מטופלים	%
20x	0.365	23.5	0.772	48.4
50x	0.266	17.2	0.712	44.6
100x	0.182	11.7	0.849	53.2
200x	0.098	6.3	0.612	38.4
דור	1.550	100%	1.594	100%



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Lowry.

#	Sample			µl Water	660nm	
1	~~B~~ B —			100	0.057	
2	BSA	10	⑧ x250	90	0.063	6
3		20		80	.078	21
4		25 30	㉓	70	.084	~~8~~27
5		40		60	71	
6		50		50	.088	
7		100		0	.082	
8	"92"	10		90	.066	
9	(1:5)	25	~~0.25~~ 0.125	75	.073	
10		50		50	.072	
11		100		0	.070	
12	"15"	10		0	.076	
13	(1:5)	25	0.125 ~~0.25~~	75	.083	
14		50		50	.070	
15		100		100	.068	
16	RAG1	10		0	.069	
17	(1:10)	25	~~1mg~~	75	.075	
18		50	250	50	.076	
19		100		100	.064	
20	481	10	0.5	90	.066	
21		25	2mg	75	.094	
22		50		50	.086	
23		100		0	.070 ~~.067~~	
24	RV1	10		90	.078	
25		25	1mg	75	.071	
26		50		50	.061	
27		100		0	.074	

~~28~~

				660nm
28	RV23-4	10	90	.074
29		25	75	.068
30		50	50	.072
31		100	0	.072
32	IgUβ	10	90	.461
33	1:10	50 ~~100~~	–	1083
34	15ado	10	90	.463
35	1:10	50 ~~100~~	–	1180

1	BSA	0
2		10
3		20
4	RAG	10
5		20
6	481	10
7		20
8	RV1	10
9		20
10	RV23	10
11		20
12		
13		

4/1/81.

	∠	µl			
92	1	20	~~.007~~	.006	
	2	40	~~.007~~	.015	.015
15	3	20		.010	
	4	40		.010	.015
RAG	5	20		.001	
	6	40		.029	.020
481	7	20		.000	
	8	40		.010	.010
RV1	9	20		.021	~~.020~~
	10	40		.014	.020
RV23	11	20		000	
	12	40		.024	.018
BSA	13	10		.022	
X ~~12~~ ㉓ ~~Et.~~	14	20		.053	→
	15	40		.123	
250µl	16	60		.162	
IgUB 1:10		10		.227	
15abs 1:10		10		.465	

~~.026 → 230~~

~~.001 → $\frac{23}{26}$ × $\frac{1}{4}$ $\frac{220}{26}$ = 8.8~~

~~= $\frac{2?}{104}$ mg.~~

~~.010 → 88 µg/ml.~~

~~.020 → 176 µg/ml.~~

IgUB

40 → 110

0.110 → 230

0.001 = $\frac{230}{110}$ = 2.1

.01 → 21 µg/ml

.02 → 42 µg/ml.

IgUB.

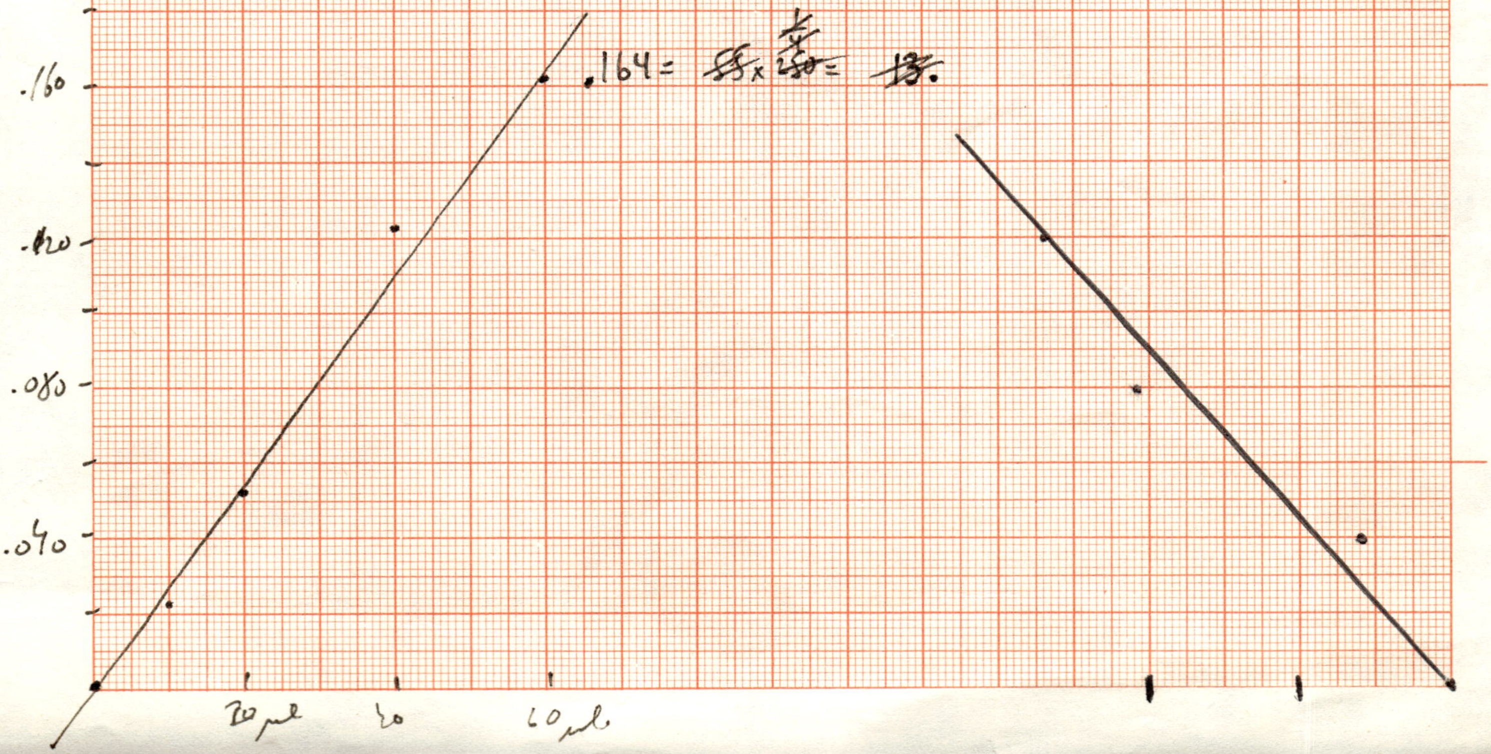
10 → .027

.027 → 230

.227 → 1934

2 mg/ml.

Lowry 4/1/82.



.164 = ~~55~~ x ~~$\frac{1/5}{250}$~~ = ~~13~~.

126
.057

100 μl.

	660	600	corr	Δ
R	137	116	137	.087
481	147	144	129	.068
RV1	141	125	.1~~3~~39	.089
RV23-4	118	095	.118	.061
92	116	101	58 .111	.054
15	126	107	.126	.069

100 μl → 0.270 for 230 μg/ml.

.270 → 130 μg/ml.

$.001 \rightarrow \frac{130}{270} = 0.5$

.054 → 27 μg/ml.

.087 → 43 μg/ml.

.061 → 30.

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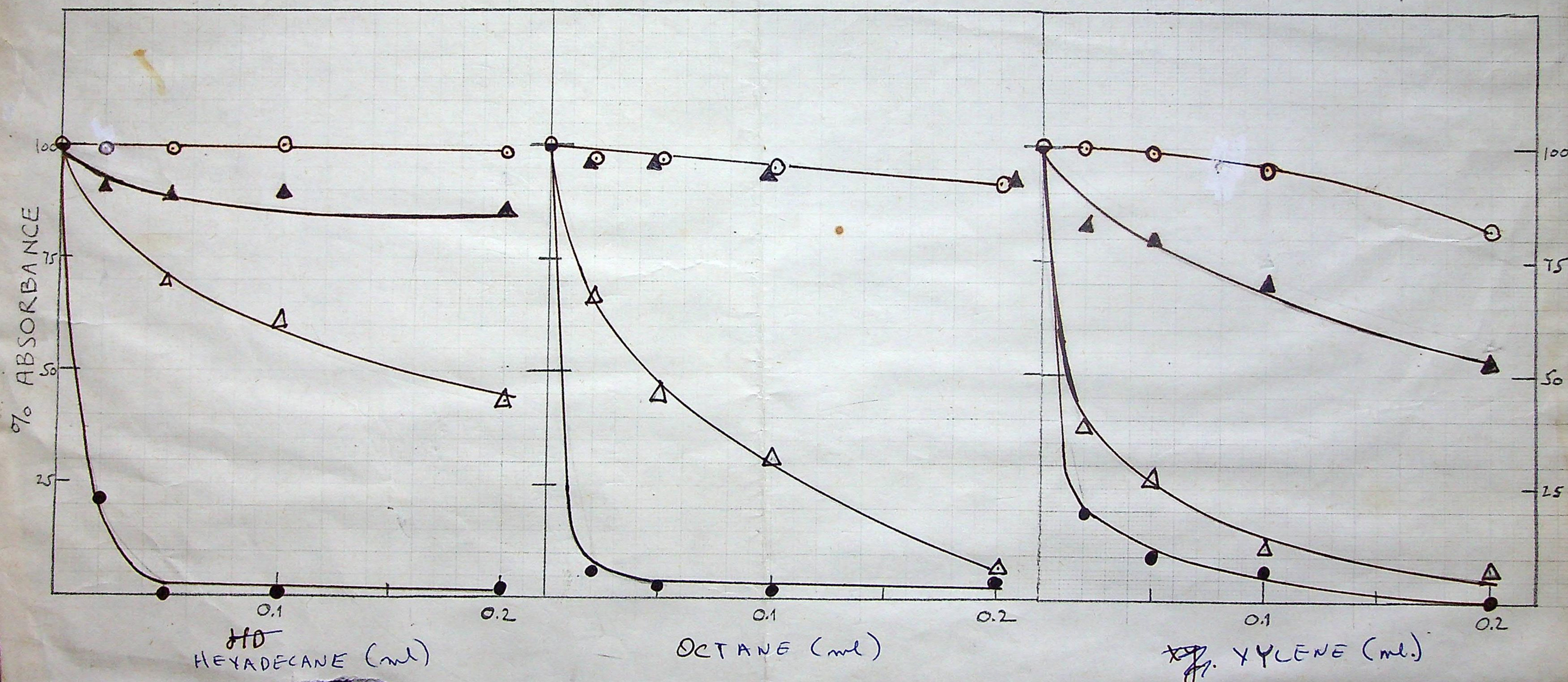
Adherence of RAG-1, MR-481, RV-1, RV-23.

~~Fig~~

22/7/80
and
29/10/80

6/10/81
and
27/10/81

1



Rag-1	1.489	(●)
MR481:	1.463	(○)
RV-1	1.556	(▲)
RV-23	1.461	(△)

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Altered cell-surface characteristics of non-agglutinating mutant of *Acinetobacter calcoaceticus* RAG-1

Acinetobacter calcoaceticus AB-15, a mutant of strain RAG-1-92 selected for its inability to be agglutinated by antibody raised against the parent strain, was studied using several techniques. Electron microscopy revealed that AB-15 was lacking thin pili, 3.5 nm in diameter, which cover the parent cells, both following growth on rich medium and minimal medium supplemented with ethanol. A crossed-immunophoretic study of cell surface components of parent and mutant cells revealed that AB-15 was missing a distinct surface antigen present on RAG-1-92 cells. These results suggest that thin pili are the major agglutination antigen of RAG-1-92 cells. In addition, mutant AB-15 exhibited altered adherence properties with respect to the parent strain. Although AB-15 and RAG-1-92 adhered similarly to liquid hydrocarbon (hexadecane) and polystyrene following growth on rich medium, AB-15 cells were non-adherent following growth on ethanol. AB-15 cells were also deficient in their ability to grow on hexadecane under conditions of limited agitation. These results suggest that thin pili contribute to the adherent properties of RAG-1-92, and play a crucial role in growth on hexadecane.

Role of Thin Pili in Adherence of *Acinetobacter calcoaceticus* RAG-1 to hydrocarbons and growth on hexadecane.

MR-481 is a cell-surface mutant of *Acinetobacter calcoaceticus* which was isolated for its inability to adhere to hydrocarbons. Non-adherent mutant MR-481 was unable to grow on hexadecane, yet prolonged incubation on hexadecane led to the isolation of two sequential clones, RV-1 and RV 23-4, which exhibited surface characteristics which differed from those of both MR-481 and RAG-1 cells. RV-1 and RV 23-4 exhibited increasing adherence to hydrocarbons, and had regained the ability of wild type RAG-1 cells to adhere to polystyrene. Wild type RAG-1, non-adherent mutant MR-481, and partial revertants RV-1 and RV 23-4 were compared for agglutination in the presence of antibodies raised against whole RAG-1 cells. MR-481 cells were not agglutinated, whereas RV-1 and RV 23-4 both agglutinated two to three times as quickly as wild type RAG-1 cells. Agglutinability of RAG-1 under the conditions studied has been attributed to the presence of thin pili (3.5 nm diameter). Upon electron microscopic examination, the thin pili which cover wild type RAG-1 cells were almost completely absent from MR-481 cells; on the other hand, strains RV-1 and RV-23 were both covered with many more pili than the wild type strain. A comparison of RAG-1 and non-adherent mutant MR-481 using crossed immunoelectrophoresis revealed that several distinct antigens are missing from the surface of the non-adherent mutant

28/10/81

Adherence of RAG-1 strains grown on BHI to the three test hydrocarbons

Rag-1, MR-481, RV-1 and RV 23-4 were tested for adherence following growth to stationary phase on BHI. Results, on accompanying figure, show a steady increase in adherence in going from MR-481 to RAG-1. In the table below, some characteristics of these cells are described; for growth on rich medium:

Strain	Thin pili (EM)	Agglutinability by IgMB	Adh to HD	Adh to PS	Φ Sensitivity AP-3	φ1	φ11	215H
Rag-1	+	+	+++	+	S	R	S	S
MR481	–	–	–	–	S	R	S	S
RV-1	++	++	+	+	S	R	R	S
RV23-4	++	++	++	+	R	S	S	S

Results suggest the following model:

1) Pili or pili-associated component can mediate adherence to polystyrene and hydrocarbons.

Evidence

(a) When MR-481 finally comes upon HD in liquid, more than 90% of colonies adhere to PS. Random study of four of these clones – all grew back thin pili.

(b) Of all the various mutants in the lab, tested, only AB-15 showed adherence deficiency. Cells grown on EtOH did not adhere to HD nor PS.

2) Adherence of RAG-1 grown in complex medium is due to the presence of several components on the cell surface, one of which is thin pili.

Evidence:

a) MR-481 lacks at least five surface antigens as shown by C.I.E.

b) ~~AB-15~~ AB-15 is conditionally adherent – adheres following growth on complex medium but doesn't have pili.

c) The transition 481 → rv1 → rv234 is accompanied at each step by differences in adherence and phage sensitivity; ~~suggesting that~~ RV-1 has grown back thin pili; RV23.4 ~~has~~ still has thin pili but also other adherent properties.

3) Adherence important for growth on HD under limited emulsification.

Evidence:

(a) MR-481 does not grow on HD (J. Bact.)

(b) Following prolonged incubation and transfers on HD, MR-481 produced ~~too~~ two successive partial revertants exhibiting increased adherence to hexadecane.

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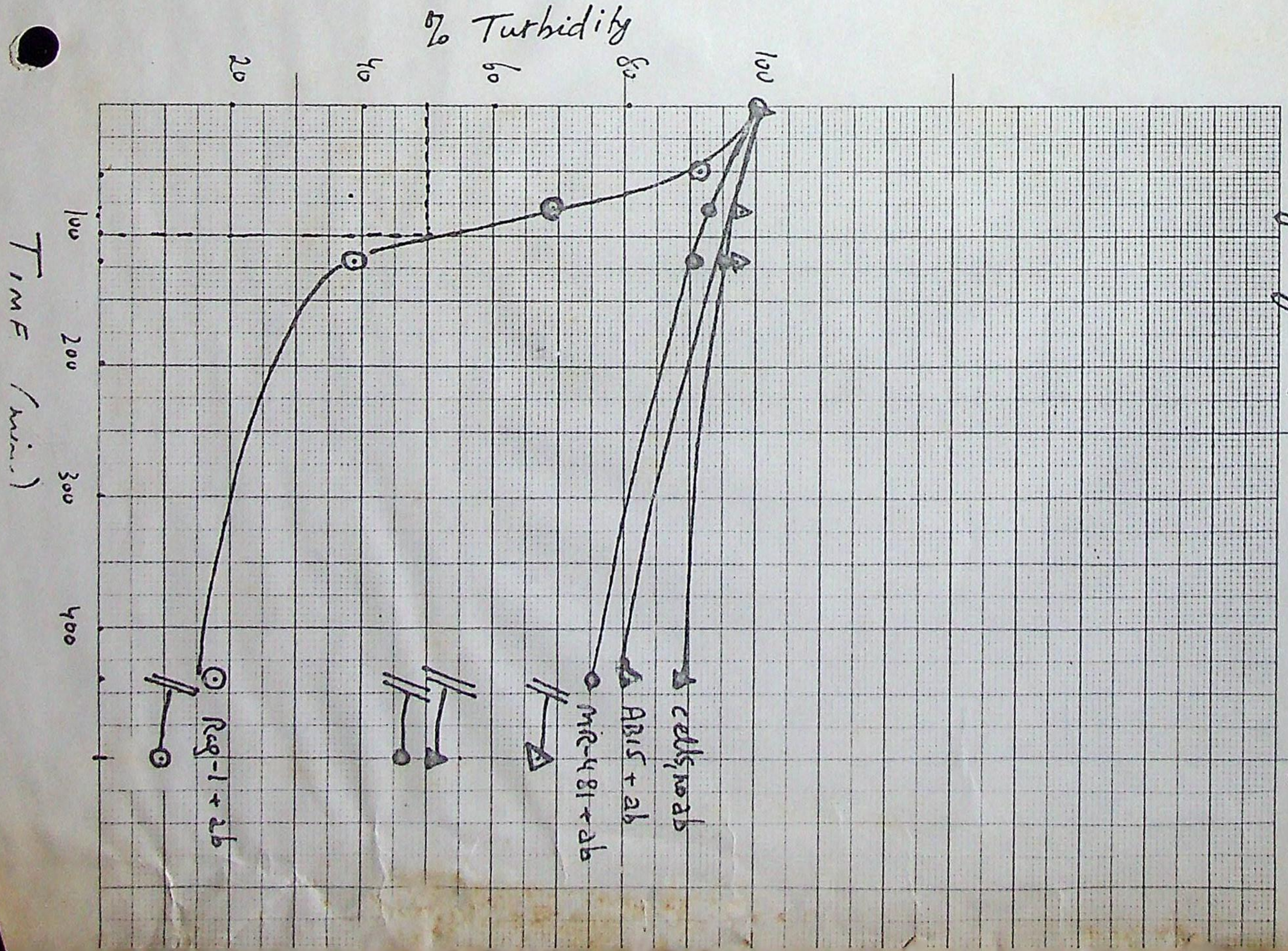
Adoor Agglutination of

and 15 by IG-UB antibodies

Cells were grown to stat. phase in brain heart, washed and resuspended in PBS to $A_{400} \sim 1.3$. To 700μl cells, 3μl of IgUB antibodies were added and agglutination followed turbidimetrically as a function of time, at room temperature

Results

Sample	Initial A_{400}	% Turbidity 25'	55'	80'	120'	440'	2880' *1
RAG-1	1.331	98	97	97	95	90	47
MR-481	1.343	99	96	97	94	89	55
AB-15	1.288	99	96	96	96	90	51
RAG-1 w/ab	1.354	99	92	69	39	18	10
MR 481 w/ab	1.382	99	96	93	91	75	46
AB-15 w/ab	1.343	99	97	97	97	81	67



Agglutination of RAG-1, MR-481 and AB-15 by IgUB (readsorbed antibodies).

19/6/81

Adherence of RAG-1, MR-481 and AB-15 to Hexadecane following growth on B.H.I.

Cells were grown to stat. phase on brain heart, washed and resuspended in PUM to A_{400} ~ 1.5-1.6

Adherence to hexadecane as always.

Results -

Sample	Initial A_{400}	% Adherence		
		50µl	100µl	200µl
Rag-1	1.502	100.	99.1	97.7
MR-481	1.613	7.2	7.2	8.7
AB-15	1.605	98.7	97.1	100.

NB - Neither MR-481 nor AB-15 were agglutinated in 70% EtOH, while RAG-1 was.

Conclusions - AB-15 are as adherent as RAG-1 following growth on brain heart, despite their inagglutinability by both adsorbed and non-adsorbed ab (and by ethanol), and their lack of observable thin filaments in the EM.

In other words, the thin filaments which are responsible for agglutination of RAG-1, are not the whole answer.

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Partial Adherence Mutants (Revertant) 3/8/81

Twenty test tubes ~~were~~, containing 4.5 ml PUM + 0.5 ml hexadecane, were each inoculated with 1.2×10^8 c.f.u./ml of MR-481. Following 72 h. incubation at 30°C with shaking, considerable growth was observed in four test-tubes:

Test-tube no.	K.U. (uncorr).
16	
17	290
18	230
19	225
20	330

Each of these test-tubes was streaked out onto nutrient agar and the colonies examined tested for adherence to polystyrene. All the colonies tested (at least 50 on each plate) from each plate did replicate onto ps. One clone was subsequently selected from each test-tube and numbered rV1, rV2, rV3 and rV4.

The inoculum and a control, containing no hexadecane were both checked for colonies adhering to ps. Of the 6000 colonies examined (inoculum) and >50 (control), none adhered to the ps.

20=5

10×2.5

The "revertant" strains were examined on July 31st for
① adherence to hexadecane
② agglutinability by 5µl of IgUB,
following 20 h. growth on brain heart infusion at 30°.

1. Adherence to HD: as usual, growth to stat. phase in BHI.

Strain	Initial O.D.	% adherence to	
		100µl	500µl
Rag-1	1.313	99	98
MR-481	1.474	0	0.3
rv 1	1.435	3.4	40
rv 2	1.462	0	11.1
rv 3	14.57	0	7.1
rv 4	14.34	0	1.3

2. Agglutinability by 5µl IgUB: To 700µl cells suspended in PUM to initial OD's as in previous expt., were added 5µl of IgUB and O.D. followed with time. Data are presented as T for O.D. to decrease by 50%

Strain	T½ (min.) (approx.)
Rag-1	115
MR-481	>>130
rv1	45
rv 2	57
rv 3	60
rv 4	42

The rv strains were sent on Aug. 2nd for EM examination.

Interim conclusions-

1. Adherence to ps and HD are not necessarily the same phenomenon. Another example is BD-4: adhered to ps but not to HD.

2. Revertants of MR-481 which have apparently regained their ability to grow well on HD are not able to adhere well to HD following growth. It appears as if they have acquired back only a portion of the adherent characteristics of RAG-1. The revertants have also regained the RAG-1 agglutinability phenotype. In fact, they are much more agglutinable than RAG-1. Have they grown the thin pili back? It appears so.

These results, taken together with the peculiar adherence characteristics of AB-15, suggest the following model:

① Adherence of RAG-1 to hydrophobic surfaces is mediated by several components. ~~These components are a~~ The expression of these components is determined by growth medium.

② Adherence to ps and adherence of cells grown on ethanol is related to the presence of pili. Thus the non-adherence of ethanol-grown 15. Thus the adherence to ps of the revertants.

③ The three or four components missing from the 481 surface (C.I.E.) do not imply a pleiotrophic ~~mutan~~ mutation, since more than one component is involved ~~in~~ adherence, and MR-481 is missing all the adherence components.

④ "Partial" adherence mutants such as the

MR481 ϕ Resistent,
482 and
rV1 - rV4

may not be screening mutants, but rather lacking one or more of several components which together contribute to RAG-1 adherence to hydrocarbons.

⑤ Adherence on B.H. and ethanol may be two different phenomena: i.e., adherence on ethanol mediated by pili, adherence on rich medium by other factor(s).

Partial Adherence Mutants - cont. 12/9/81

3. EM - Rag-1, MR-481 and RV-1-4 were negative stained (2% uranyl acetate) following 20h. growth on BHI. All four revertants were very heavily pilated (thin), compared to RAG-1.

4. Growth on ethanol - 19/8 - Cells were grown in PUM + 2% EtOH and harvested following 48h and 72h and tested for adherence to ~~0.2 ml~~ HD.

48h

Strain	Growth Kn corr	Initial O.D.$_{400}$	% adherence to 50µl	100µl	200µl	Aggl. O.n.
Rag-1	580	1.487	74	83	98	+
481	430	1.537	4.4	4.0	3.8	−
rV-1	430	1.430	3.3	5.5	4.9	−
rV-2	430	1.548	9.2	11.0	14.0	−
rV-3	320	1.394	5.0	4.6	4.5	−
rV-4	390	1.470	5.2	3.5	3.5	−

72h

Strain	Growth Kn corr	Initial O.D.$_{400}$	% adherence to 50µl	100µl	200µl	Aggl. O.n.
Rag-1	460	1.316	−	−	96	+
481	460	1.299	−	−	8.7	−
rV-1	460	1.296	−	−	20	+
rV-2	460	1.186	−	−	22	+
rV-3	460	1.241	−	−	10	+
rV-4	460	1.241	−	−	17	+

5. RV-1: Adherence to three test hydrocarbons -

800ml. RV-1 cells were grown in BHI to stat phase, (20h) washed twice ~~and suspended in~~ in $H_2O \times 1$, and resuspended in PUM for adherence to hydrocarbons. Bulk of the cells were resuspended ~~H~~ in 15ml H_2O and frozen for further testing. See also figure next pg.

Initial O.D.: 1.527

µl	% adherence to		
	HD	OC	XY
50	92	–	81
100	92	106	71
200	93	95	57
500	92	–	46

6. Growth of RV-1 vs. Rag-1 and MR481 on hexadecane

The growth of RV-1 was compared with growth of RAG-1 and MR-481 on hexadecane as in the J. Bact. paper, starting at an initial O.D. of 5 K.U.

Controls:

(1) 0.2% acetate. Following 1-2 h lag, cells grew with a doubling time of ~50 min

2) No carbon source: no growth obsewed

3) Experimentals:

test-tubes 1-3 : Rag-1
4-7: MR-481
8-10: RV-1

	Test tube	Initial K.U.	9h	23h	31h	48h
RAG-1	1	5	27	69	70	–
	2	5	10	65	66	–
	3	5	13	57	59	–
MR-481	4	5	4	4	4	47
	5	5	7	6	9	64
	6	5	10	9	10	24
	7	5	5	4	5	17
RV-1	8	5	5	4	9	70
	9	5	5	3	5	35
	10	5	5	6	17	70

(4) Adherence to hexadecane. At the outset of the expt., inocula were tested for adh to hexadecane. RAG-1 adhered while neither MR-481 nor RV-1 did. Rag-1 and RV-1 adhered to P.S. while 481 did not.

Following expt (48h) two test tubes of each strain were tested for adherence to HD following growth on HD. Results:

Strain	Initial O.D.	% Adh to 0.2ml HD	Aggl. w/ IgUB
Rag-1	1.420	89%	+
MR-481	1.412	6.5%	+
RV-1	1.616	8.7%	+

Cells were also tested following 24h. growth on acetate.

Strain	Initial O.D.	% Adh. to 0.2ml HD.	Adh to p.S.
Rag-1	1.083	89	+
MR-481	1.449	4.4	−
RV-1	1.334	14	+

(5) Adherence to P.S.

From each (MR-481) HD test-tube, plates of N.A. were streaked and the plates tested for ~~growth~~ adherence to p.s. (replica method). Results:

# Test-tube plated out	Adh to P.S.
481 (acetate grown)	0/>100
# 4	40/40
5	at least 42/50
6	39/40
7	40/40

7. ϕ-typing - Preliminary results of phage-typing:

Strain / Phage →	ap2	ap3	#8	#11
Raf-1	–	–	–	–
MR 481	–	–	–	–
RV1	+	–		
RV2	+	–		
RV3	+	–		

Conclusions

1. When MR-481 is inoculated into PUM+HD under the conditions studied, growth takes place after a lag of several days. The majority of cells isolated from the growth culture are ~~pard~~ ~~partial~~ revertants to w.t. in the following ~~propert~~ properties:

a) adherence to polystyrene (both washed cells and colonies)

b) agglutination by specific antibodies (IgUB)

c) thin pili (E.M.)

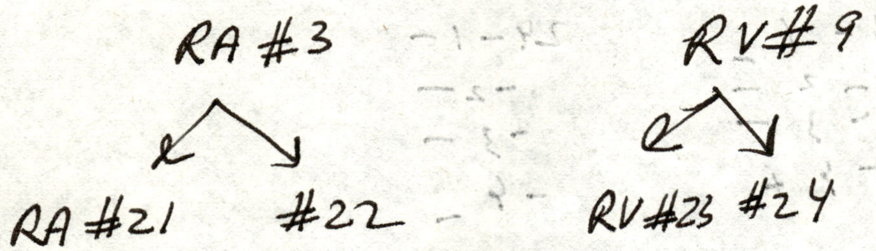
They have also gained resistance to ϕ ap-2, similar to the phenotype of MR-482, isolated last year, which is partly deficient in adherence to HD.

However, these cells do not exhibit significantly greater adherence to hexadecane than 481, following growth on various media (BHI, ethanol, acetate, hexadecane). Revertants were not obtained following growth of 481 on these other media.

2. When revertant cells were compared with MR-481 and RAG-1 for growth on HD, a similar lag was observed for RV-1 and MR-481 tubes. However, the cells from the MR-481 tubes that grew were no longer 481 cells but themselves revertants!.

Oct 5/81

Growth tubes RAG-1 #3 and RV-1 #9 served as inocula (0.1 ml into 4.2 ml) for new HD growth test-tubes as follows:



~~After four as days growth:~~

Growth:

	48h.	96h	144h.	Adh to HD
RA-21	30	33	35	~~0.700~~
RA-22	8	48	60	
~~RA~~ RV-23	43	45	60	
RV-24	45	56	68	

Following 144 h growth, ~~5~~ 3 ml removed from each tube, pooled washed and tested for aggl. and Ad. to HD.

	Init O.D.	% Ad to 200µl HD	t½ agglutination
RAG	0.700	98.7	~1½ h
RV	0.560	41.3	~2 h

RV22 and 23 were plated onto NA and four isolated colony selected from each. These were tested qualitatively for adh to HD.

Results

23-1	–	24-1	–
-2	–	-2	–
-3	–	-3	–
-4	+	-4	–

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Comparison of properties of RA6-1, MR481, RV-1 and RV23-4 following growth on ethanol

5/11/81

One ml of inoculum from a PUM+2% EtOH (v/v) culture was inoculated into 50 ml. of PUM + 2% EtOH (v/v) in 250 ml. flasks and cells were grown at 30°C for 48 h. in Gutnick's shaker. Cells were spun down, washed twice and suspended in PUM. The following treatments were performed on cells/sups:

1) adherence to hydrocarbons
2) agglutination by IgUB antibodies
3) growth on hexadecane
4) presence of thin pili (neg. stain)
5) emulsifying activity of undialyzed sups
6) omnimixing of cells.

Results-

A. Growth (klett units)

RA6-1 : 980
MR-481 : 950
RV-1 : 1120
RV23-4 : 630

B. Emulsifying activity of non-dialyzed sups –

(0.2 ml into 7.5 buffer + 0.1 mixture)

Strain	K.U.	Corr K.U.	Activity/ml	Activity/KU growth/ml
RAG-1	250	313	117	0.119
MR-481	230	288	108	0.113
RV-1	265	351	132	0.117
RV23/4	175	200	75	0.119

C. Adherence to Hydrocarbons.

Strain	Initial O.D.	HD (μl)			OC (μl)			xy (μl)		
		50	100	200	50	100	200	50	100	200
RAG-1	1.452	65	66	82	41	86	99	47	61	88
MR481	1.455	20	22	41	25	27	31	19	21	47
RV-1	1.428	19	22	32	0.8	29	26	41	61	86
RV23-4.	1.415	20	27	42	44	49	48	42	77	90

D. Agglutination by IgUB.

5 μl of IgUB antibody were added to 700 μl cells suspended in PUM to A_{400} of 1.0-1.25

Strain	Initial O.D.	$t_{1/2}$ (min.)
Rag-1	1.129	~100
MR-481	1.186	~120 (!)
RV-1	1.222	~60
RV-23-4	1.214	~80

E. Growth on hexadecane.

Cells were washed twice and suspended in PUM to 100 K.U. Suspensions were then diluted 1:20 into test-tubes containing 4 ml PUM + 0.5 ml Hexadecane (exptls), or PUM + 0.2% EtOH (controls).

Results-

1. HEXADECANE

Test-tube	TIME (h). Growth (K.U.)				
	0h.	29h.	31h	46h	73h.
RA6-1	5	0	0	4	39
RA6-1	5	0	1	7	40
481	5	3	1	6	35
RV-1	5	5	9	10	23
RV23-4	5	10	18	28	34

2. Control: 0.2% EtOH

	0h.	11h	13h	26h
RA6-1	5	3	17	231
481	5	1	38	208
RV-1	5	25	45	204
RV23-4	5	85	138	208

F. Presence of thin pili: From E.M., all cells had lots of thin pili except for 481, which had <5 per cell.

Comparison of Properties 5/11/81

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25/1/81

HD-Adherence of HD-grown cells of RAG-1 and mutant MR-481

Cells of RAG-1 and MR-481 were grown ~~4d.~~ on 10% (v/v) hexadecane in test-tubes at 350 r.p.m, 30°C. After 4 d. when turbidity in both RAG-1 and mutant tubes was ~120 K.U., cells were spun down, ~~[illegible]~~ washed and resuspended in PUM. Adherence to 200 µl HD as usual.

Results -

	RAG-1	MR-481
Before	0.900	1.079
After	0.096	0.956
% Adherent	89.3	11.4

Conclusions -

1. Growth of MR-481 on HD is not due to revertants.
2. The aqueous fraction of RAG-1 cells is adherent, even though in the aqueous phase.
3. Under the circumstances, adherence is not necessary for growth on hexadecane i.e., mutant cells were able to grow.

14/10/81

Adherence of H10N

Acinetobacter calc. strain H10N was tested for adherence to hexadecane following growth overnight in Brain heart infusion broth. Cells were washed twice, + suspended in PUM. Adherence was tested as usual.

Results obtained (with RAG-1 added for comparison):

	H10-N	RAG-1
Control:	1.411	1.489
HD (μl):		
20	97.7	82.0
50	96.8	100.0
100	98.6	100.0
200	98.6	97.2

Conclusion: H10-N more adherent than RAG-1.

Table 1. Effect of Periodate Treatment and Sugar Addition* on adherence of late-exponential RAG-1 ~~cells~~ to Epithileal cells.

Addition	Adherence (% of control)
None**	100
None (periodate treated)***	40
mannose	40
α-methyl-D-mannose	54
β-D-glucose	98
methyl-β-D-glucoside	86
D-glucosamine	107
n-acetyl glucosamine	116
D-galactose	88
methyl-α-D-galactoside	112
maltose	100
L-arabinose	100

* Adherence was performed in the presence of various sugars at a final concentration of 2.5%

** In the control 43% of the epithelial cells scored ~~to~~ had 100 or more attached bacteria

*** Epithelial cells were incubated 15 min. in the presence of 10 mM sodium meta periodate as previously described( ).

Table 1. Effect of Sugars and Periodate Treatment on Adherence of *A. calcoaceticus* RAG-1[a] and *E. coli*[b] to ~~Human~~ Epithelial Cells.

Inhibitor tested	Adherence (% of control)	
	RAG-1	E. Coli
None	100[c]	100[d]
D-mannose	40	10
methyl-α-D-mannopyranoside	54	5
D-glucose	98	>90
methyl-β-D-glucoside	86	n. d.
D-glucosamine	107	n.d.
N-acetyl-D-glucosamine	116	85
D-galactose	88	99
methyl-α-D-galactoside	112	>90
maltose	100	>90
L-arabinose	100	n.d.
oxidation of epithelial cells with Sodium metaperiodate	40	0

---

[a] Cells were grown to late ~~log~~ exponential phase on nutrient broth as described in Materials and Methods.

~~[b] Data taken from ( ).~~

[b] Data taken from ( ).

Adherence of 92 and 15 grown on hexadecane plates

28/10/81

92 and AB-15 were grown for 3 days on $\frac{PUM}{2}$ +100 µg/ml lysine supplemented with hexadecane vapours (no growth -lysine; no-growth -HD). Plates scraped, washed twice and suspended in PUM. Adherence and agglutination as yesterday.

1) Adherence

Strain	Initial O.D.$_{400}$	% Adherence to HD (µl)	
		50	200
92	1.167	75	89
AB-15	1.180	76	83

2) Agglutination: (700 µl of cells in PUM + 5 µl IgUB)

Strain	Initial O.D.	t½ (min)
92	1.167	~130
15	1.180	>1200 (20 h.)

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5/8/80

Isolation of Non-Adherent Mutants of RAG-1

Non-adherent mutants of RAG-1 were obtained by the following technique - 50 ml. of a stationary phase culture of RAG-1 ~~were~~ in Nutrient broth (30°C, aeration) were harvested and resuspended in 5 ml PUM buffer. 2-5 ml octane was added and following mixing and phase separation the bottom phase was transferred to another test tube. 2 ml octane was added and the process continued until no cells were microscopically visible in the aqueous phase. 50 ml. of ~~nutr~~ nutrient broth were inoculated with the final lower phase and when the cells reached stationary phase this whole process was repeated. When the fourth nutrient broth culture was spun down, resuspended in PUM and octane added, turbidity remained in the lower phase even after several mixings and transfers. The aqueous phase was streaked out and three colony-types were observed:

481-B. Colonies resembling RAG-1 on NB and EtOH plates
482-S. Similar colonies, but slower growing. After several days incubation at 30°, dense inner circles surrounded by translucent mucoid growth.
483-T. Tiny colonies on NB and EtOH. Large colonies ~~ca~~ on LB plates.

Microscopic observation - 481-B and 482-S appear similar to RAG-1. While 483-T is a diplococcus, it appears in irregular clumps and does not resemble RAG-1 in appearance.

Following isolation of these three distinct types, they were grown 18h. in brain heart infusion broth (bhib) at 30°C w/ shaking, and assayed for adherence, with RAG-1 as control.

Results- (% adhering cells; initial O.D. in parentheses)

Hexadecane vol. (μl)	RAG-1 (1.563)	481-B (1.375)	482-S (1.576)	483-T (1.479)
20	97.4	0	62	0
50	95.6	0	69	0
100	94.6	0.6	65	0
200	—	3.4	68	0

Following the adherence assay the (same) cells were checked for (1) phage sensitivity and (2) agglutination with antibody.

Results -

1 Phage sensitivity
(top agar method §; repeated twice)

STRAIN:	ϕ2	ϕ3
RAG-1	S	S
481-B	S	S
482-S	R	S
483-T	R	R
312	S	R
201	R	S

Agglutinability - Assayed by microscopic observation; three concentrations of antibody; compared with cells alone and cells + serum:

Strain	Agglutinability by "95"	Agglutinability by "5"
RAG-1	++	++
481-B	$++^{L}$	+++
482-S	$+^{L}$	++
483-T	–	–

Cells from these liquid culture were streaked onto plates; then bhib cultures were ~~not~~ reinoculated from these plates and adherence assayed again:

HEXADECANE VOL (µl)	RAG-1 (1.322)	481-B (1.351)	482-S (1.540)	~~483-T~~
50	98.5	0	~~30~~ 30	
200	–	27	22	

Conclusions - These experiments established that 481·B and 482·S are indeed mutants of RAG-1 and are deficient in ability to adhere to hexadecane ~~under~~ when grown under conditions which promote adherence in the wild type. 481-B appears to be wholly deficient and 482-S partially deficient. 483-T appears to be a contaminant.

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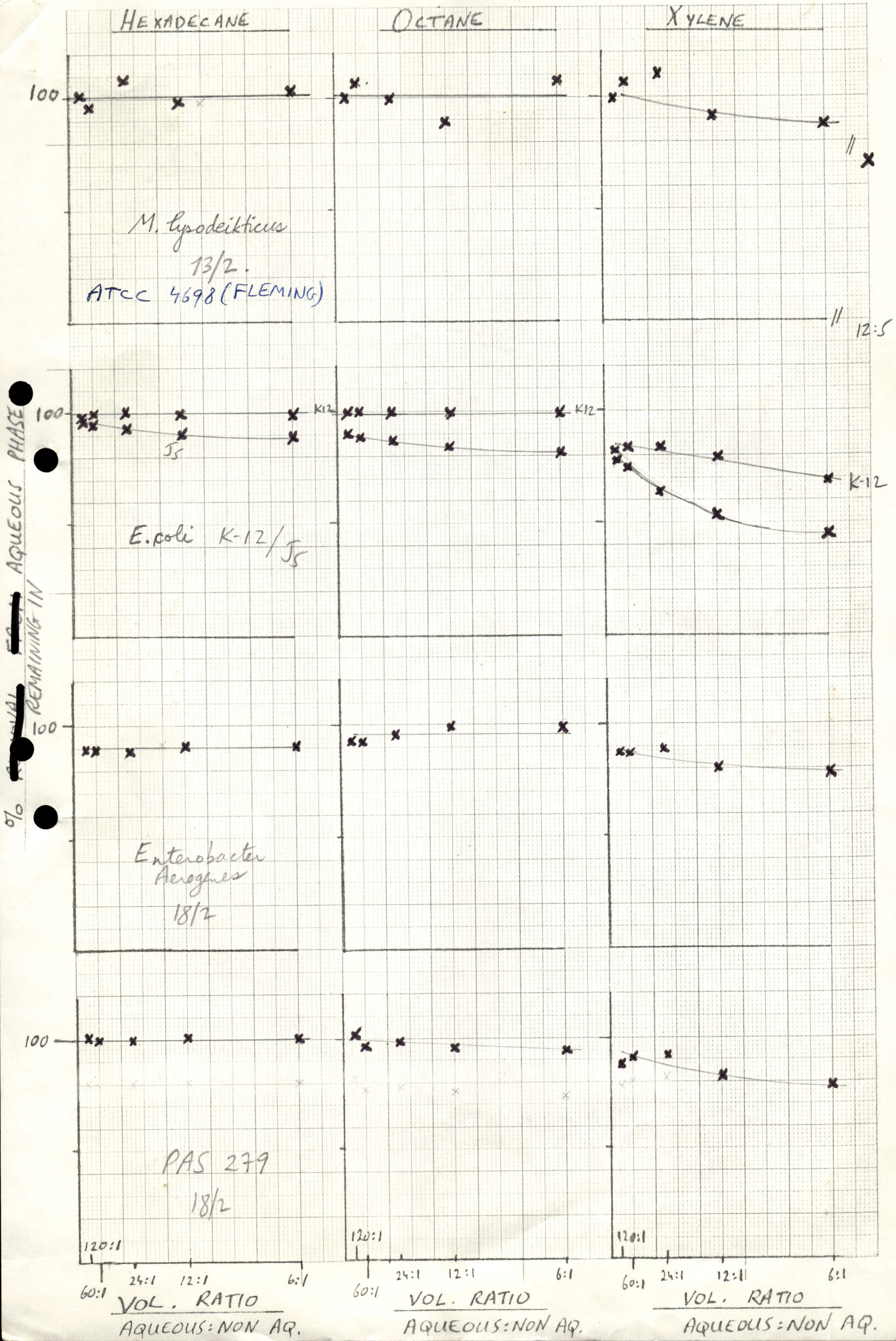
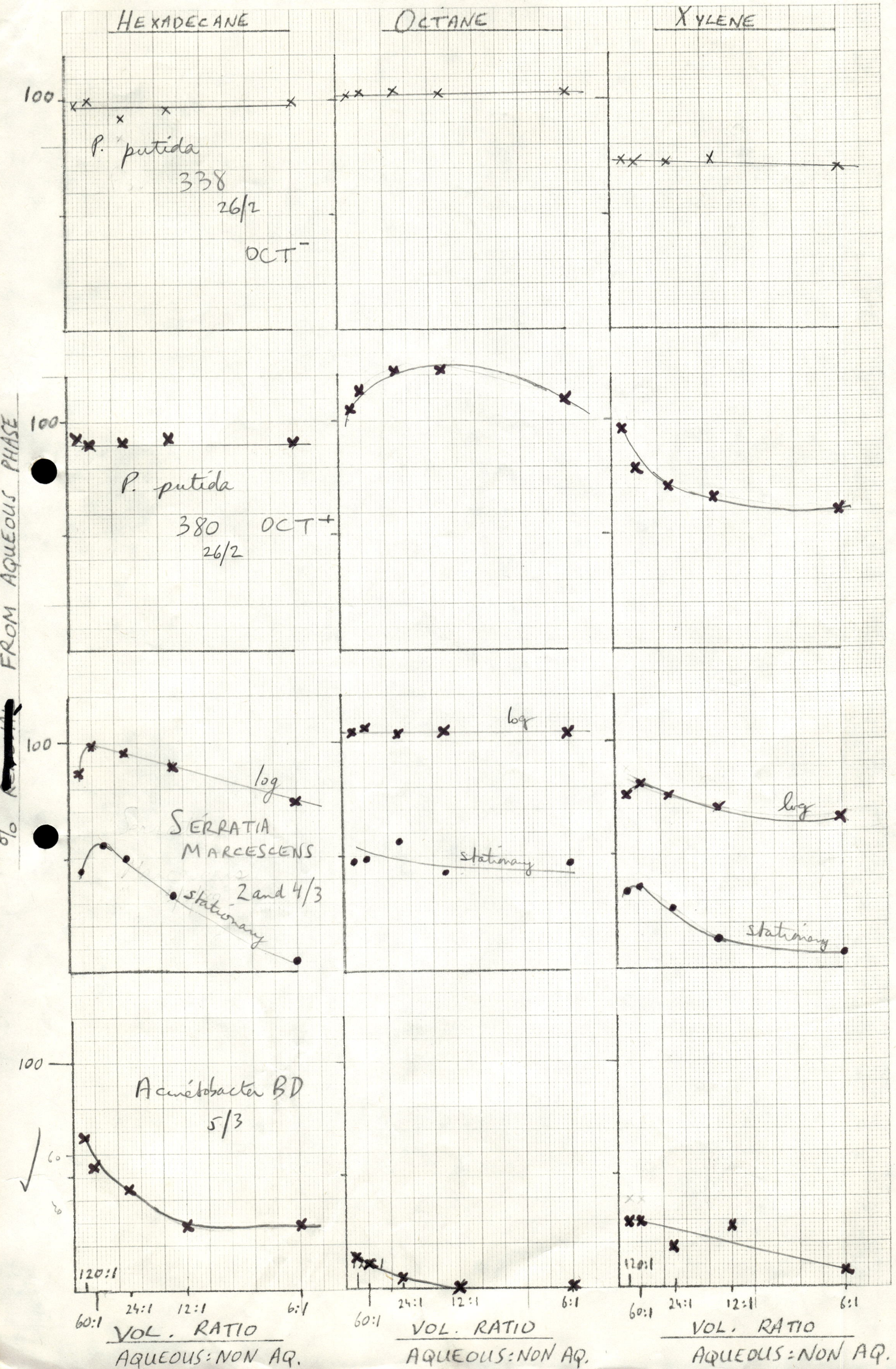
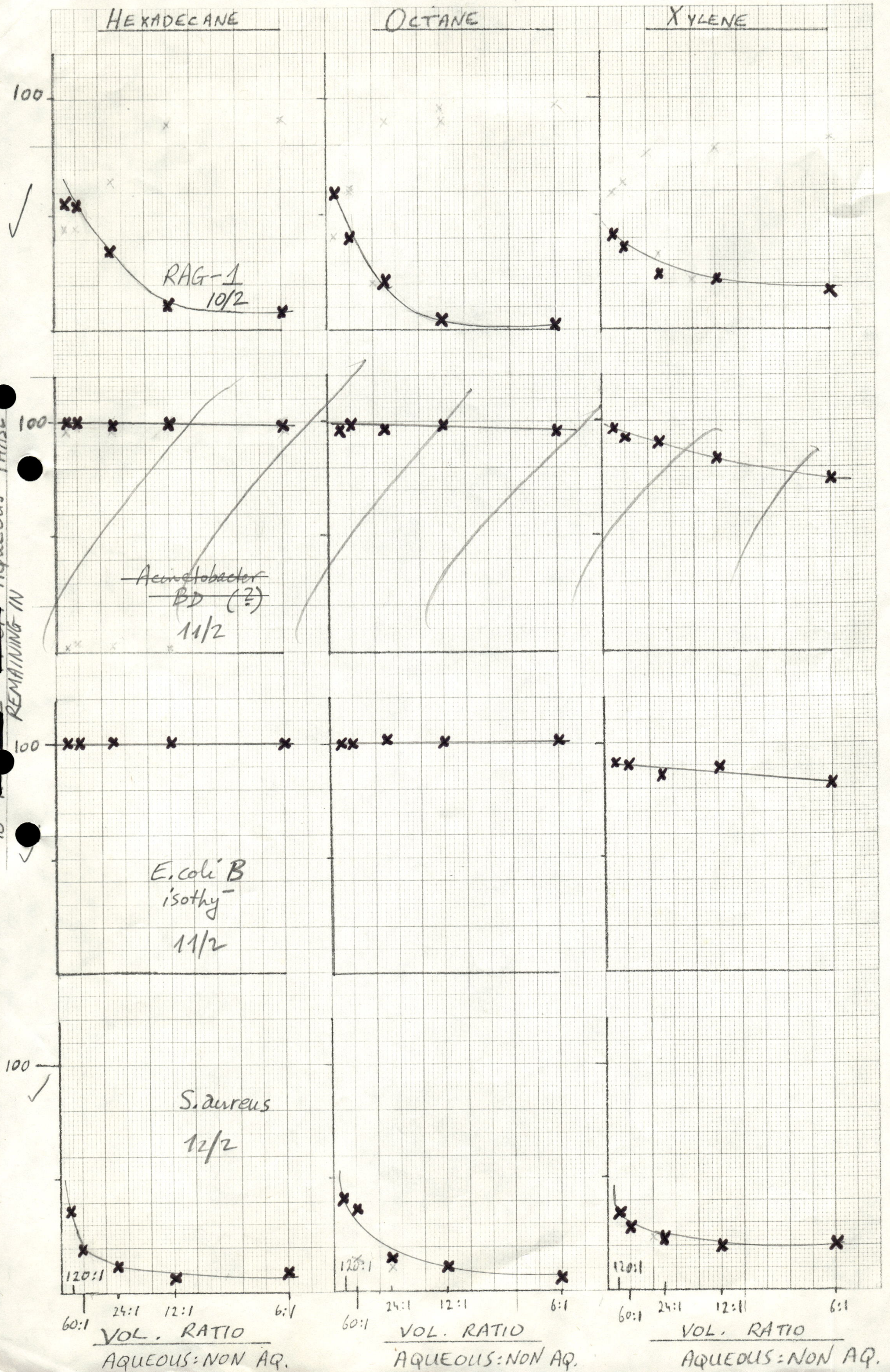
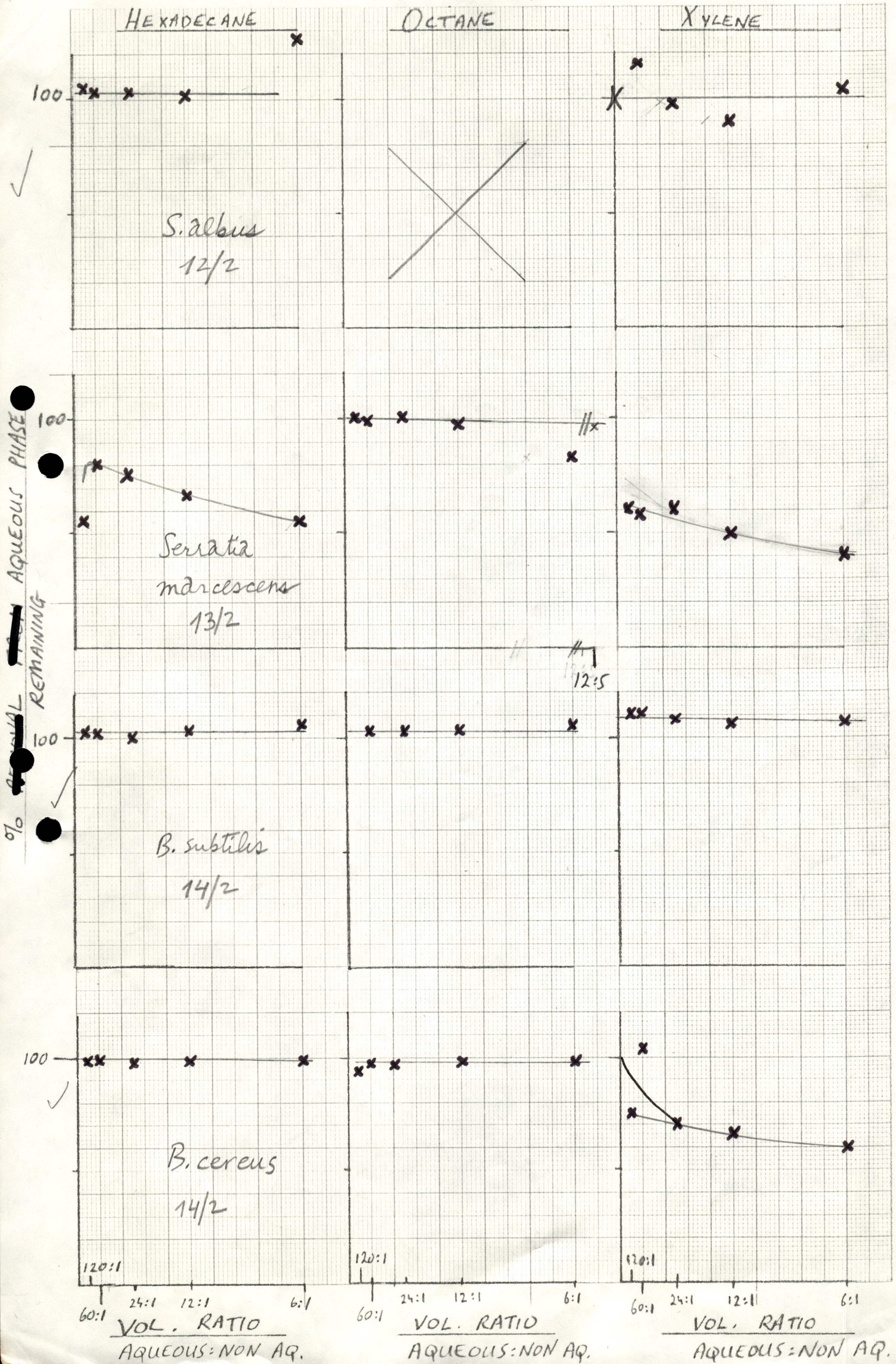
10 - 18/2/80.


HEXADECANE
OCTANE
XYLENE
% REMAINING IN AQUEOUS PHASE
100
M. lysodeikticus
13/2.
ATCC 4698 (FLEMING)
12:5
K12
K12
J5
K-12
E. coli K-12/J5
Enterobacter Aerogenes
18/2
PAS 279
18/2
120:1
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS : NON AQ.


HEXADECANE
OCTANE
XYLENE
100
P. putida
338
26/2
OCT⁻
100
P. putida
380 OCT+
26/2
100
log
SERRATIA
MARCESCENS
2 and 4/3
stationary
log
stationary
log
stationary
100
Acinetobacter BD
5/3
60
20
120:1
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS: NON AQ.
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS: NON AQ.
120:1
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS: NON AQ.
% FROM AQUEOUS PHASE


HEXADECANE
OCTANE
XYLENE
% ~~REMOVAL FROM~~ AQUEOUS PHASE
REMAINING IN
100
RAG-1
10/2
~~Acinetobacter~~
~~BD (?)~~
11/2
100
E. coli B
isothy⁻
11/2
100
S. aureus
12/2
120:1
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS : NON AQ.
120:1
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS : NON AQ.
120:1
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS : NON AQ.


HEXADECANE
OCTANE
XYLENE
100
S. albus
12/2
Serratia marcescens
13/2
B. subtilis
14/2
B. cereus
14/2
12:5
%
AQUEOUS PHASE
REMAINING
120:1
60:1
24:1
12:1
6:1
VOL. RATIO
AQUEOUS: NON AQ.

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26/6/81

Adherence of Et-grown cells

Cells were grown 48h. on PUM + 2% EtOH (v/v) + lps (100 µg/ml), harvested, and washed twice and resuspeded in PUM. Adherence to HD as usual (with addition of 0.5ml).

Cells-Control.	50 µl	% turbid	100 µl	%	200 µl	%	500 µl	%
# 15:1.445	1.427	98.8	1.351	93.5	1308	90.5	.993	68.7
# 2:1.393	.557	40.0	.370	26.6	.264	19.0	.205	14.7
# R:14.77	.790	53.5	.681	46.1	.575	38.9	.301	20.4
# 481:1.370	1.358	99.1	1.361	99.3	1.242	92.7	1.098	80.9

Growth (0.3 in 5.0 ml total; K.U.)

		TOTAL KU.
15	50	833
92	71	1183
Rag-1	70	1167
481	85	1417

Agglutination in 75% EtOH

15	–
92	++
Rag-1	++
481	–

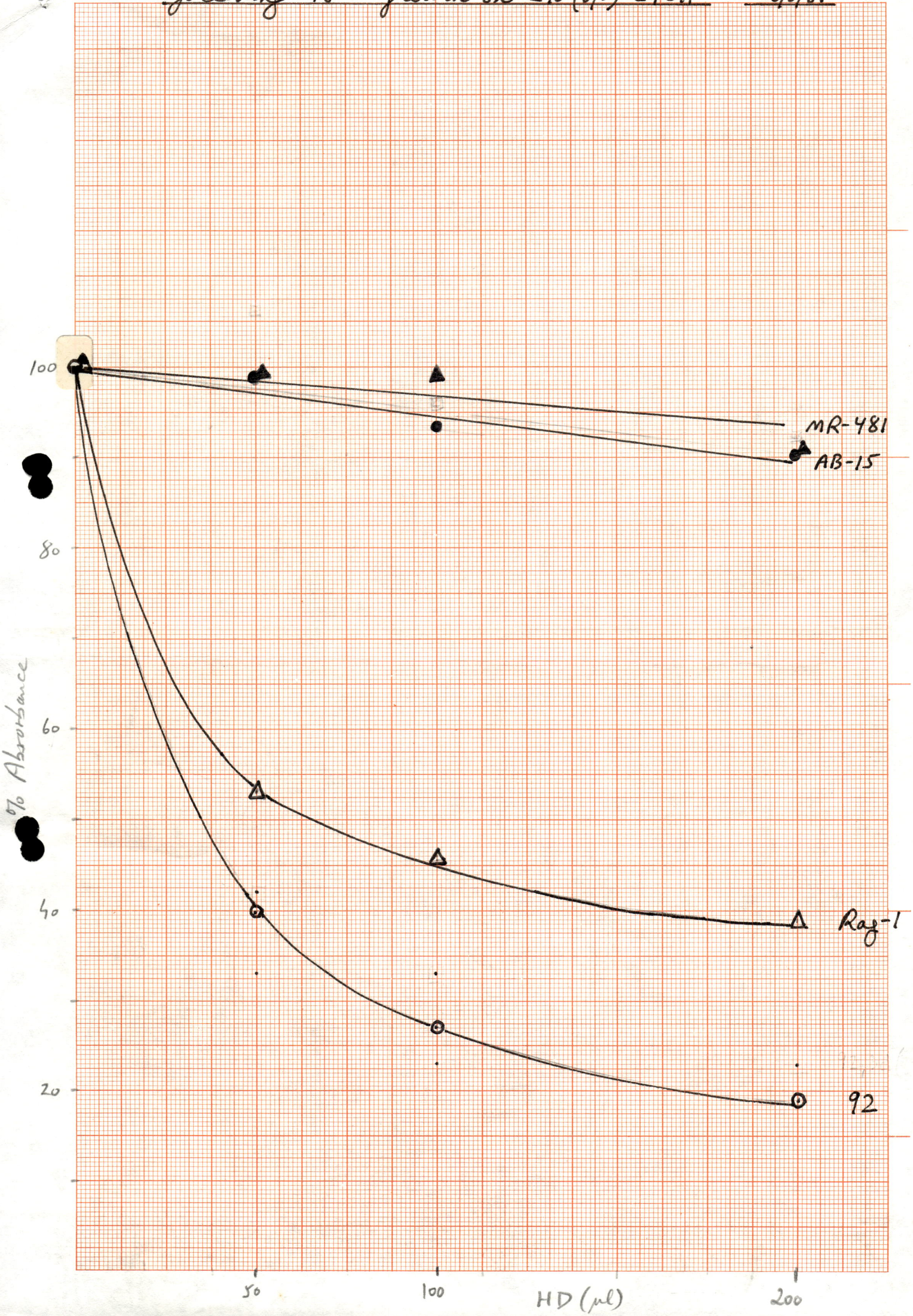
29/6/80

72 h. cultures of 92 and 15.

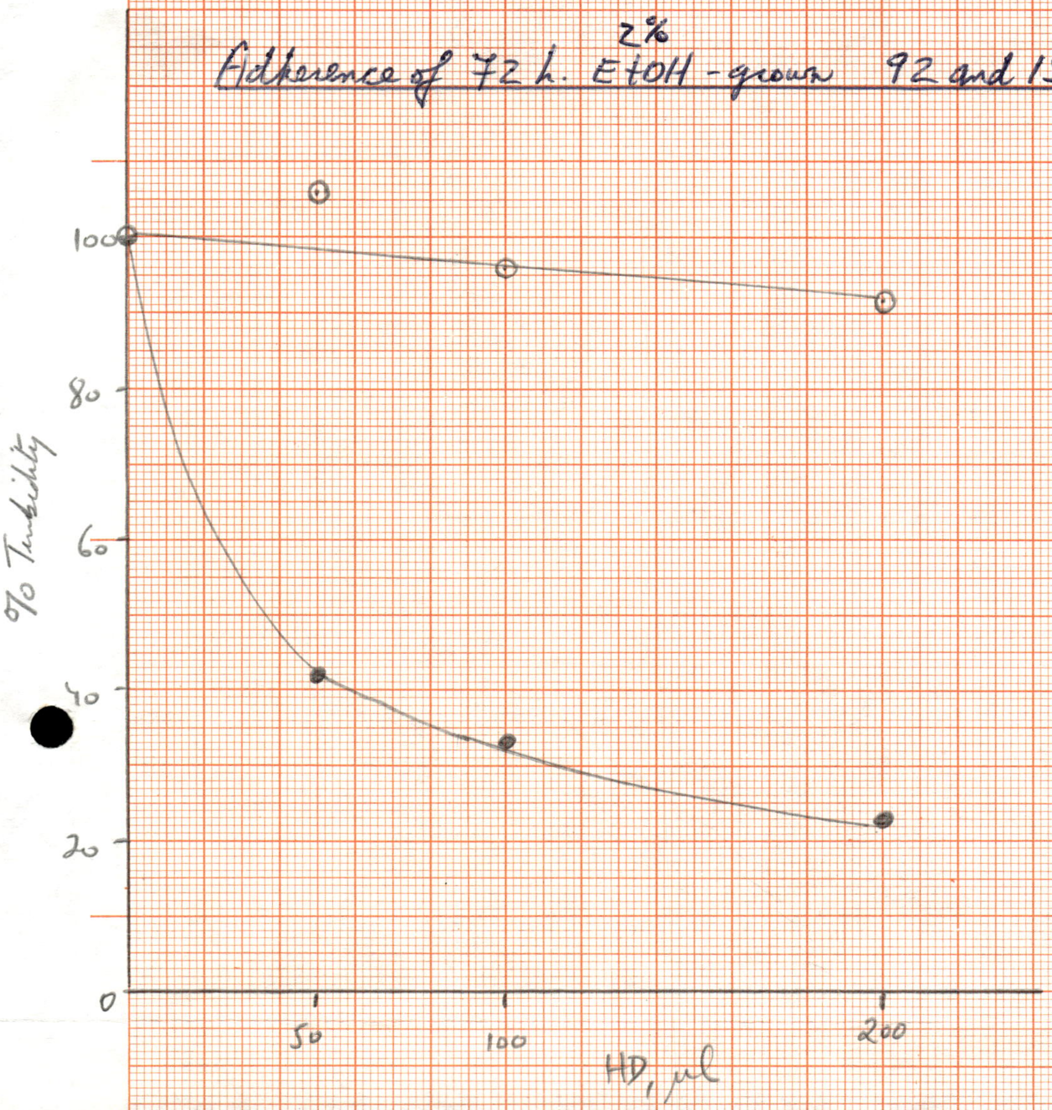
92 – 1.363 }
15 – 1.388 } initial O.D.

Both agglutinate in EtOH, 15 AB was agglutinated by UG-IB (see book)

Adherence to hexadecane of mutants MR-481 and AB-15 following 48 h. growth on 2% (v/v) EtOH 26/6/81



29/6/81



Adherence of 72 h. 2% EtOH-grown 92 and 15 cells to HD.

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Growth on Hexadecane 3/1/81

Details: Same as in previous expts:

- Test-tubes 1 and 2, containing 0.5 ml hexadecane were vortexed for 30 sec. at time zero.
- Test-tube 3 also contained 0.5 ml HD, but was not vortexed initially.
- Test-tube 4 contained 0.05 ml HD and was vortexed for 30 sec at time zero.
- Test-tube 5 : - Carbon source
- Test-tube 6 : - PUM + 0.5% EtOH
- Test-tube 7 : - brain heart infusion broth

Initial cell concentration:

	Rag-1	MR-481
Turbidity:	10 KU	10 KU
c.f.u.	9.4×10^7	7.1×10^7
Total (coulter)	6.7×10^7	5.9×10^7

Growth conditions: 360 r.p.m, 30°C.

Observations and Qualitative Results - 1. In the test-tubes R-1 and R-2, stable droplets were formed by initial vortexing and turbidity in the aqueous phase dropped during the first hours of growth. No droplets were observed with 481 until the second day of the expt. At 1245 min (~21h), test-tube 2 was cooled and thawed to release cells. Turbidity of R-2 (corr K.U.) rose from 238 to 259, indicating that about 10% of the cells were released. No change in test-tube 481-2 with cooling and thawing. On ~~second~~ third day of experiment, turbidity began to appear in mutant test-tubes 1 and 3.

Growth Data. (in K.U. corr.)

TIME (min)	RAG-1							MR-481						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
0	10	10	10	10	10	10	3	10	10	10	10	10	10	3
35				6										
45	0	3	10		11			14	19	11	9	7		
120	1	4	11	2	9	9		14	14	14	14	5	18	
125							16							20
180	0	4	10	4		16	59	13	12	13	12		20	61
200							85							88
240							120							121
265	10	14	10	9		34		12	11	11	12		24	
315	26	22	12	16	8	63		12	12	12	12	9	33	
440	78	77	22	37	4	162		13	11	11	12	7	37	
505	107	111	41	51		188		9	12	13	15		44	
560	128	147	60	63				12	13	14	12			
740	190	238	154	97	2	298		12	12	12	15	7	78	
1245	207		225	165	2	462		11		13	20	6	304	
1365	195		238	164				10		12	19		318	
1605	194		235	165	1			10		11	19	5	390	

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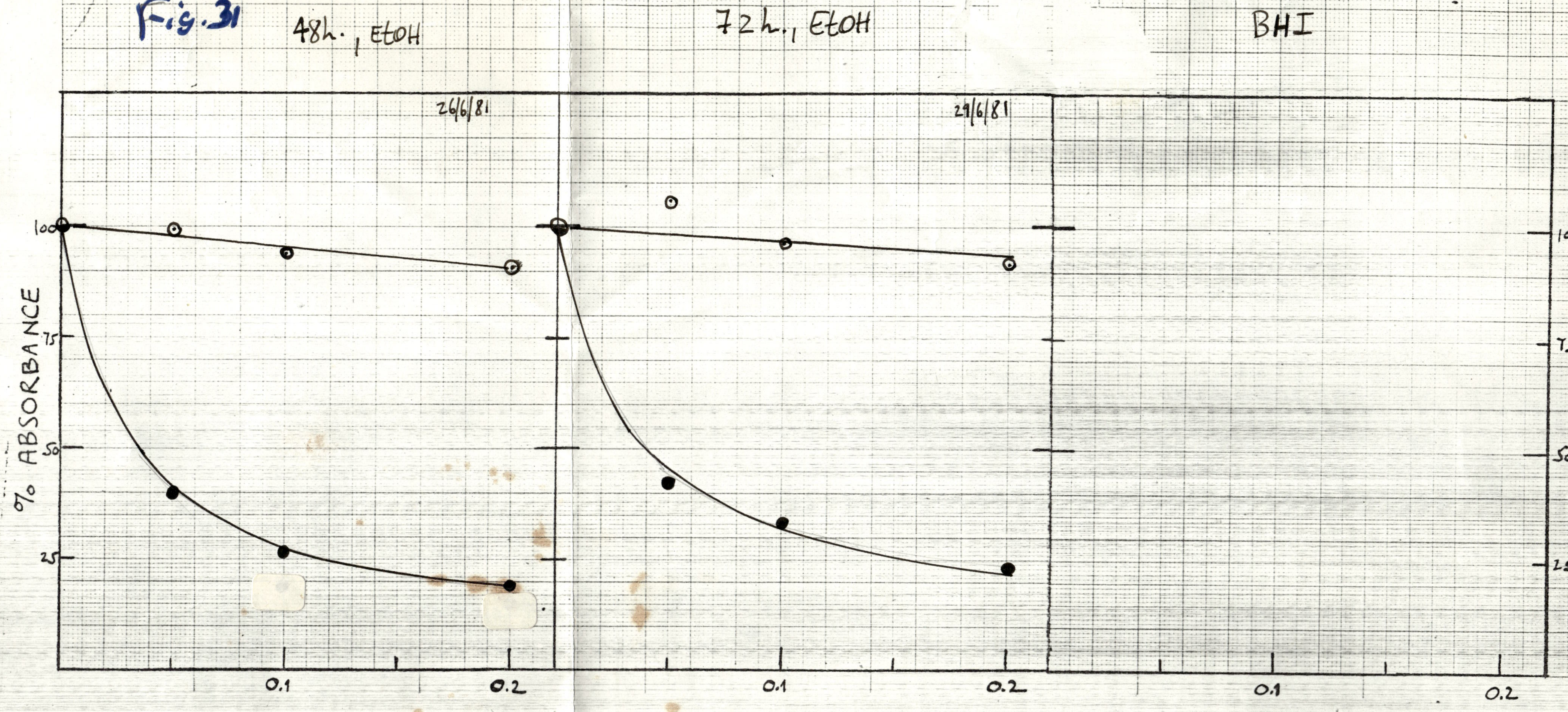
Adherence of AB-15 and RAG-92 to hexadecane

Fig. 31



15- 1.445
92- 1.[illegible]3

15-1.388
92-1.363

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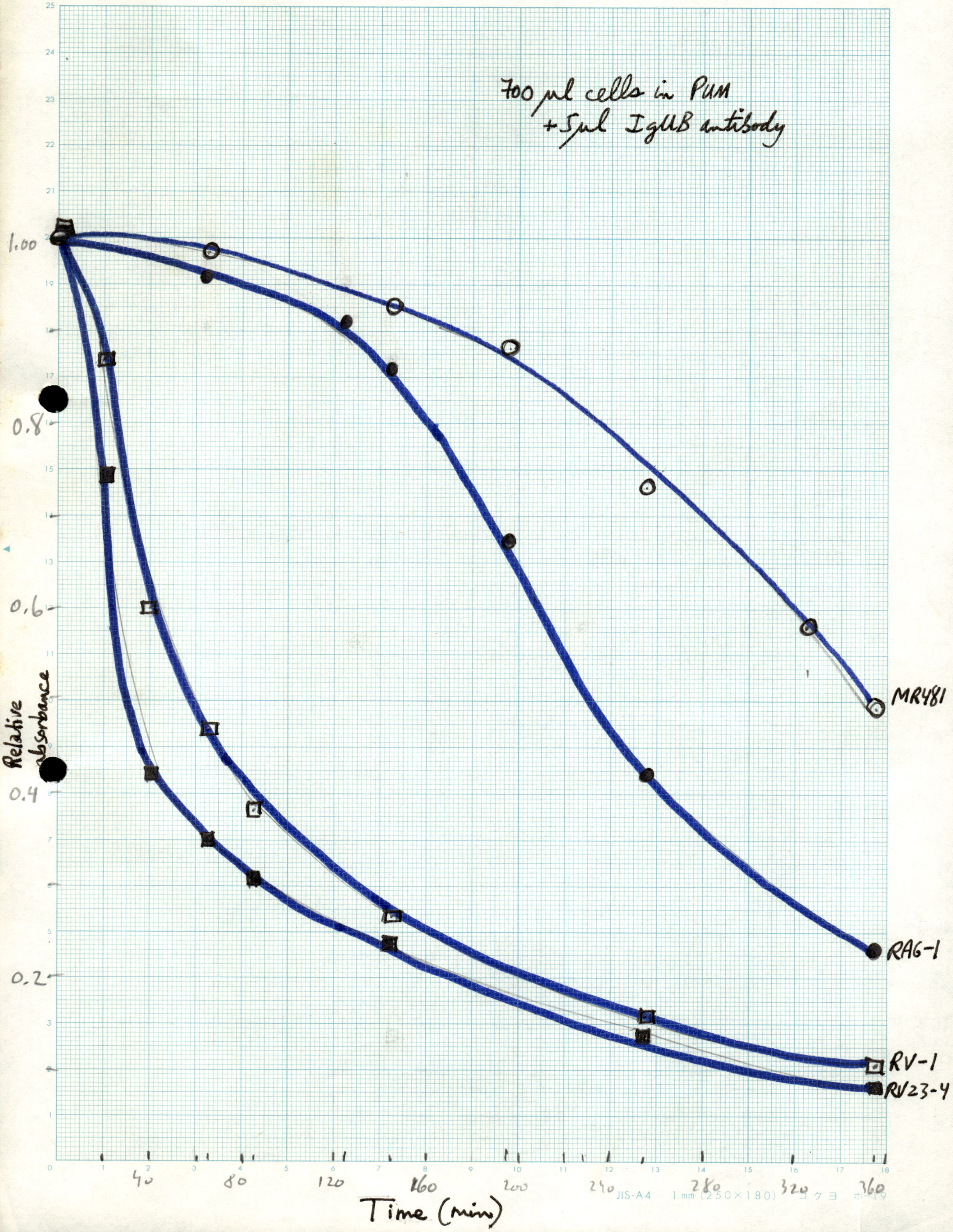
Agglutination of RAG-1, MR-481, RV-1, RV-23-4, following growth on BHI 11/11/81


700 µl cells in PUM
+5µl IgUB antibody

JIS-A4 1mm (250×180) コクヨ ホ-19

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Comparison of properties of RA6-1, MR481, RV-1 and RV23-4 following growth on ethanol

5/11/81

One ml of inoculum from a PUM + 2% EtOH (v/v) culture was inoculated into 50 ml. of PUM + 2% EtOH (v/v) in 250 ml. flasks and cells were grown at 30°C for 48 h. in Gutnick's shaker. Cells were spun down, washed twice and suspended in PUM. The following treatments were performed on cells/sups:

1) adherence to hydrocarbons
2) agglutination by IgUB antibodies
3) growth on hexadecane
4) presence of thin pili (neg. stain)
5) emulsifying activity of undialyzed sups
6) omnimixing of cells.

Results-

A. Growth (Klett units)

RA6-1	:	980
MR-481	:	950
RV-1	:	1120
RV23-4	:	630

B. Emulsifying activity of non-dialyzed sups-

(0.2 ml into 7.5 buffer + 0.1 mixture)

Strain	K.U.	Corr K.U.	Activity/ml	Activity / KU growth/ml
RAG-1	250	313	117	0.119
MR-481	230	288	108	0.113
RV-1	265	351	132	0.117
RV23/4	175	200	75	0.119

C. Adherence to Hydrocarbons.

Strain	Initial O.D.	HD (µl)			OC (µl)			xy (µl)		
		50	100	200	50	100	200	50	100	200
RAG-1	1.452	65	86	82	41	86	99	47	61	88
MR481	1.455	20	22	41	25	27	31	19	21	47
RV-1	1.428	19	22	32	0.8	29	26	41	61	86
RV23-4.	1.415	20	27	42	44	49	48	42	77	90

D. Agglutination by IgUB.

5 µl of IgUB antibody were added to 700 µl cells suspended in PUM to A_{400} of 1.0-1.25

Strain	Initial O.D.	$t_{1/2}$ (min.)
Rag-1	1.129	~100
MR-481	1.186	~120 (!)
RV-1	1.222	~60
RV-23-4	1.214	~80

E. Growth on hexadecane.

Cells were washed twice and suspended in PUM to 100 K.U. Suspensions were then diluted 1:20 into test-tubes containing 4 ml PUM + 0.5 ml Hexadecane (exptls), or PUM + 0.2% EtOH (controls).

Results -

1. HEXADECANE

Test-tube	TIME (h). Growth (K.U.) 0h.	29h.	31h	46h	73h.
RA6-1	5	0	0	4	39
RA6-1	5	0	1	7	40
481	5	3	1	6	35
RV-1	5	5	9	10	23
RV23-4	5	10	18	28	34

2. Control: 0.2% EtOH

	0h.	11h	13h	26h
RA6-1	5	3	17	231
481	5	1	38	208
RV-1	5	25	45	204
RV23-4	5	85	138	208

F. Presence of thin pili: From E.M., all cell[illegible] lots of thin pili except for 481, which had <5 [illegible] cell.

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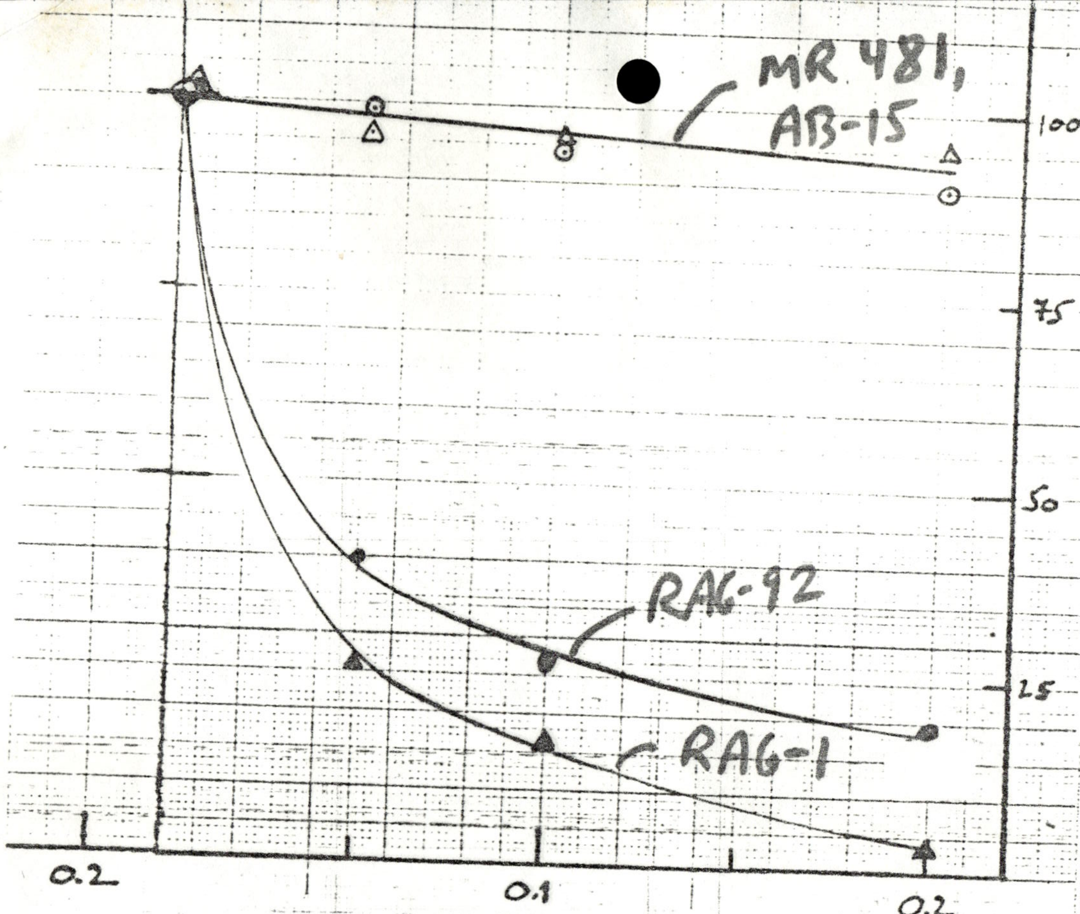
Cell Surface Properties of RAG-1 STRAINS related to adherence and growth on HC's

Strain	% Adherence to 0.2 ml HD	% Adh to 0.2 ml XY	Growth on HD vapour	Growth in HD tube	Thin Fimbriae (EM)	Agglutination ($T_{1/2}$)	Phage Sensitivity			
							215H	ϕ11	AP-3	ϕ1
RAG-1	96	95	++	+++	++	~22	S	S	S	R
MR-481	2	18	++	–	–	>>48	S	S	S	R
RV-1	14	48	++	+	+++	<6	S	R	S	R
RV23-4	57	92	++	++	+++	<6	S	S	R	S
RAG-92	100	n.d.	++	+	++	~22	S	S	S	R
AB 15	95	n.d.	++	–	–	>>48	S	S	S	R

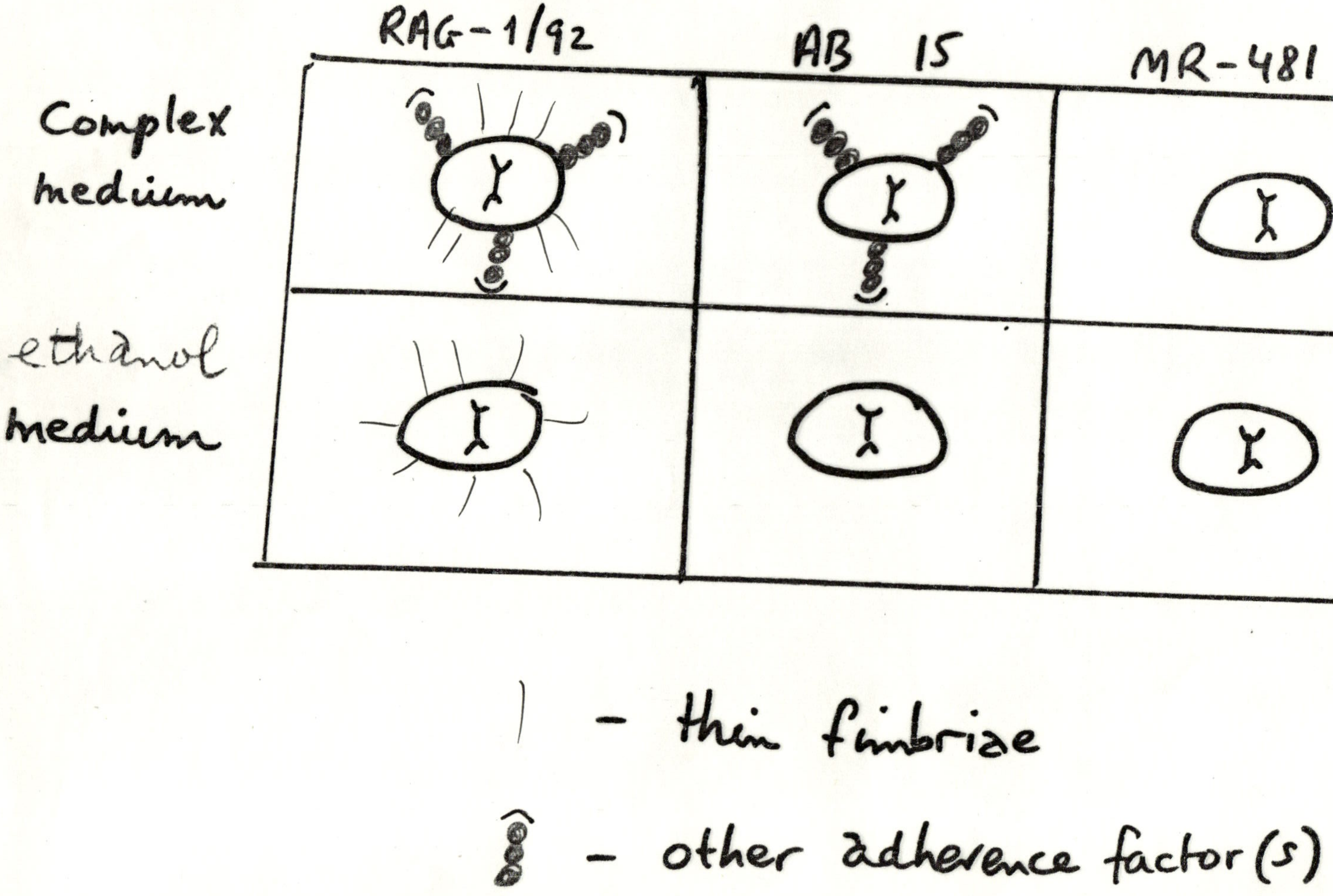


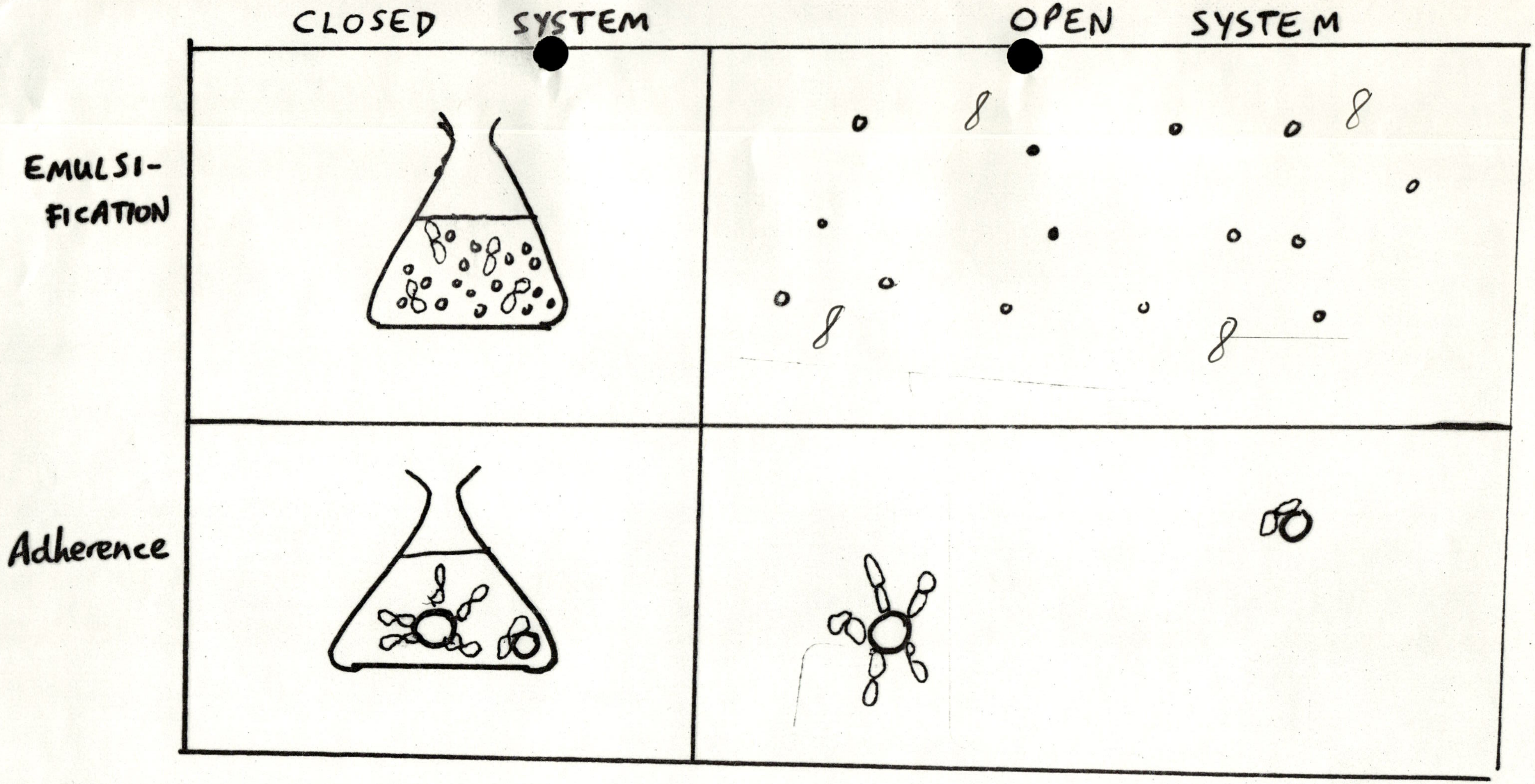
EtOH gran.

1.35–1.55

19/8/81

26/6/81


RAG-1/92
AB 15
MR-481
Complex medium
ethanol medium
- thin fimbriae
- other adherence factor(s)


CLOSED SYSTEM
OPEN SYSTEM
EMULSI-FICATION
Adherence

CONCLUSIONS:

1. Adherence to hydrocarbons is a measure of cell surface hydrophobicity, rather than a specific property of hydrocarbon-degrading bacteria.
2. Adherence to hydrocarbon is crucial for growth under conditions of (i) low initial cell density, and (ii) limited emulsification
3. Thin fimbriae play a role in adherence of RAG-1 to hydrophobic surfaces and growth on hexadecane.

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Growth data of 92 and 15 on hexadecane 8/11/81

Strains 92 and AB-15 were tested for growth on hexadecane following growth on:

(1) brain heart infusion (stat. phase, 20h.).
(repeated twice). 4/10, 2/11
(2) plates supplemented with hexadecane vapours 25/10

1. Brain heart infusion - 4/10. into PUM + 20μg/ml lysine + 0.5ml

	HD	TIME (H). (KU-uncorrected)				
		0	20	49	72	118
Ag	92	5	15	27	95	228
	92	5	13	56	78	118
	92	5	13	45	108	173
AB	15	5	13	13	14	12
	15	5	13	7	10	9
	15	5	14	12	16	72

	Acetate (0.2%).	TIME (min.).										
		0	135	260	315	345	375	405	465	495	550	5
Ag	92	5	8	28	53	66	85	102	134	152	180	19
AB	15	5	5	5	5	5	9	18	46	71	106	12

		1260.
Ag	92	218
AB	15	218

2. Brain heart - 2/11/81 PUM + 50 µg/ml lysine + 0.5 ml HD

HD	Time (hr).				
	0	23	42	72	140
Rag 92	5	5	20	30	48
Rag 92	5	5	42	35	68
AB 15	5	6	4	5	5
AB 15	5	6	6	8	–

0.2% Ac	Time (hr).					
	0	3	4.25	6.	7	16
Rag 92	5	13	23	57	70	98
AB 15	5	9	16	41	60	93

Control - PUM + 0.2% Ac, no lysine.

after 6 days: Rag-92: 3 K.U.
AB-15: 8 K.U.

3. Pregrown on $\frac{PUM}{2}$ plates + HD vapours + 100 µg/ml lysine 25/10
into PUM + 50 µg lys + 0.5 ml HD

HD	Time (hr).					
	0	24	49	61	71	88
g 92	5	3	6	57	54	50
3 15	5	6	4	6	3	5

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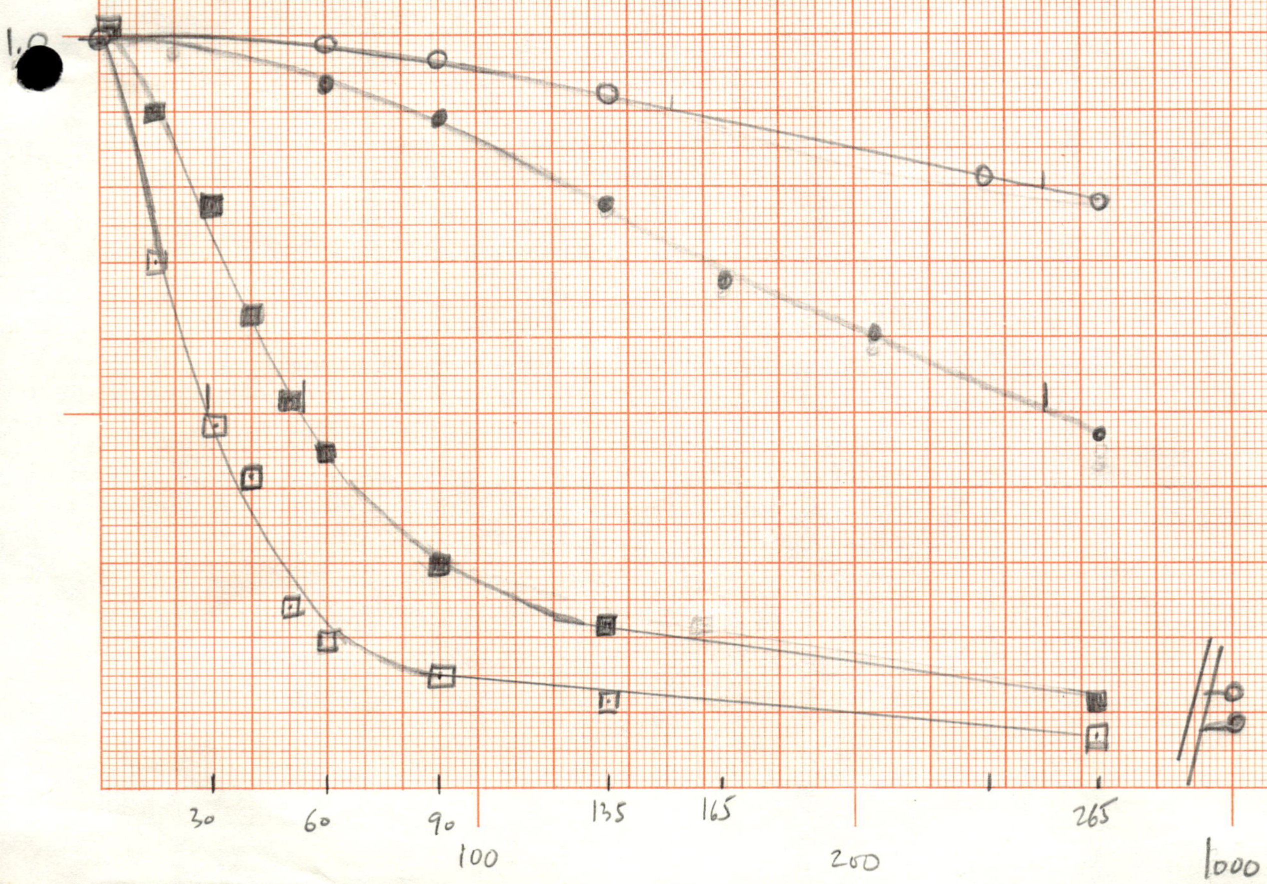
Agglutination of RAG-1, 481, RV1, RV23-4 10/11/81

$t_{1/2}$ (min).

Rag-	250
481-	400
RV1-	54
RV23-	28



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Effect of omnimixing on the adherence of EtOH (2%) grown 16 Hu to hexadecane

15/9/81

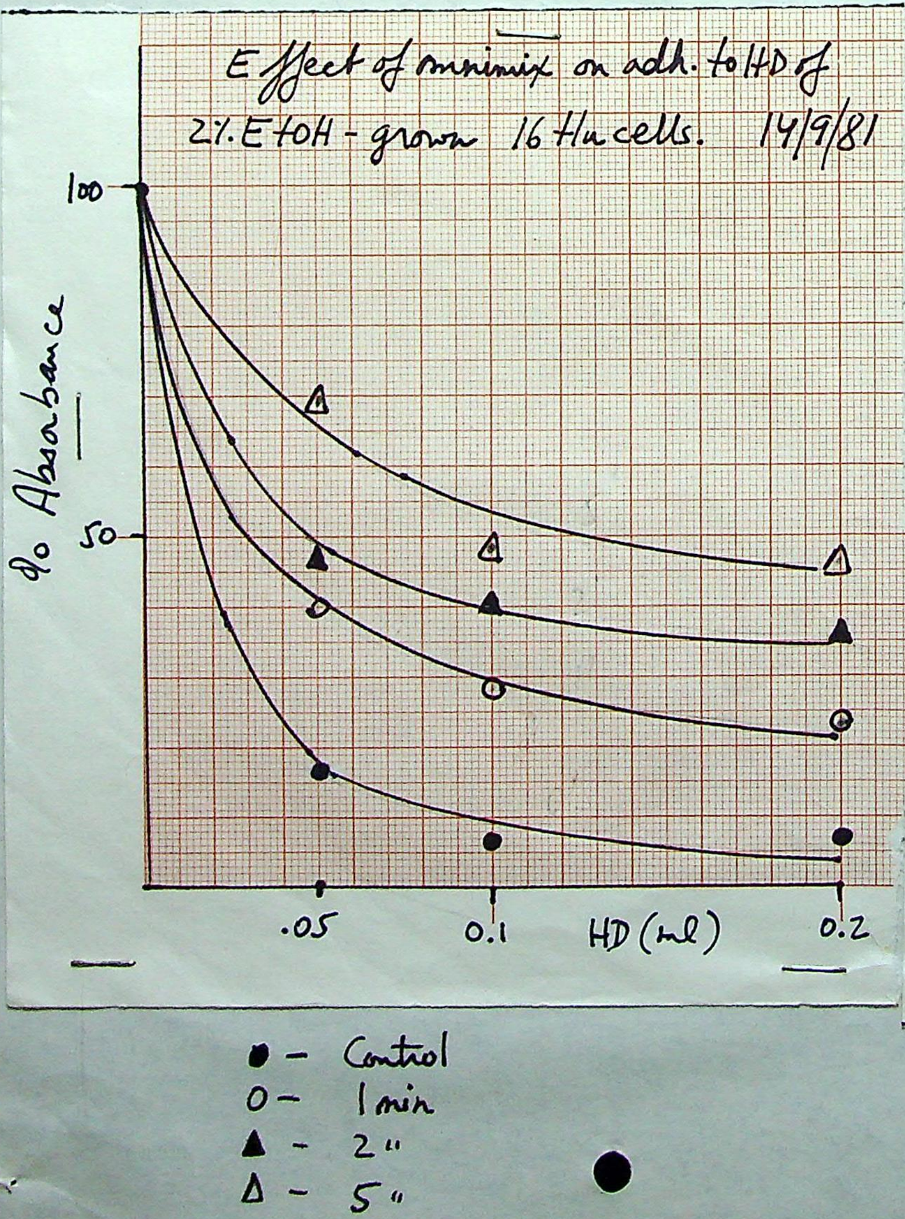
Cells of 16 Hu were inoculated 1:400 into PUM + 2% (v/v) EtOH + 100 μg/ml lysine and grown 48 h. to early stationary phase (650 K.U.). Cells were harvested, washed twice and resuspended to O.D.$_{400}$ ~ 1.4, and omnimixed at 0°C for various times at "9" on dial. 6 ml aliquots were centrifuged in the cold, and adherence to HD performed immediately following resuspension of cells in PUM. Supts. maintained at 0°C.

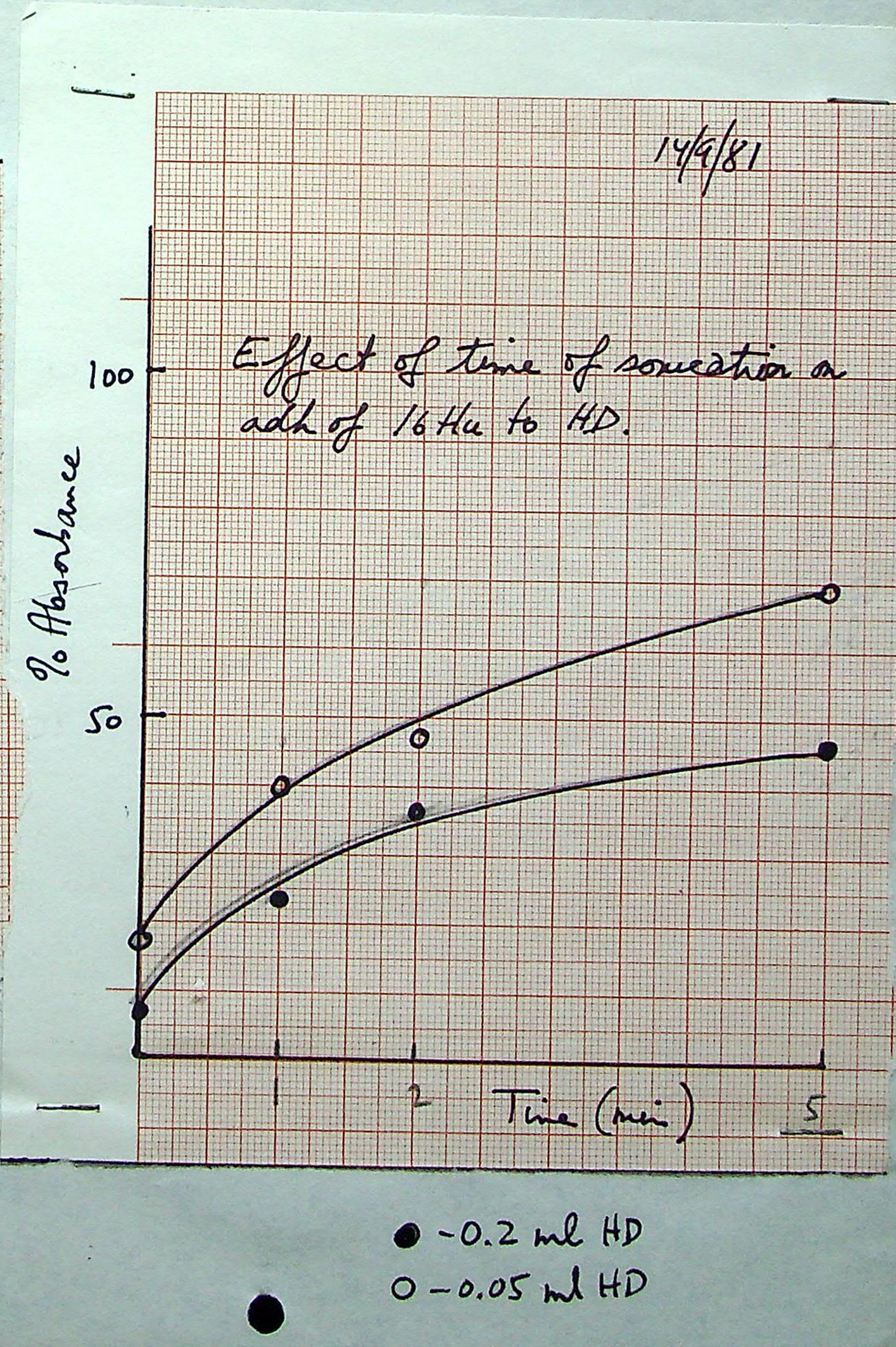
Results -

1. Adherence

TIME OF OMNI	O.D.	% Adh to HD (μl)		
		50	100	200
0	1.233	83	93.4	93.0
1	1.273	60	72	77
2	1.353	53	60	64
5	1.422	31	52	54

2. Agglutination - 1ml Cells were incubated o.n. in presence of 5μl of IgUB, at 0°C. Complete agglutination obseved in all test-tubes.


Effect of omnimix on adh. to HD of 2% EtOH-grown 16 Hu cells. 14/9/81


14/9/81
Effect of time of sonication on adh of 16 Hu to HD.

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herence of RAG-1 strains grown on BHI to the three test hydrocarbons

Rag-1, MR-481, RV-1 and RV 23-4 were tested for adherence following growth to stationary phase on BHI. Results, on accompanying figure, show a steady increase in adherence in going from MR-481 to RAG-1. In the table below, some characteristics of these cells are described; *for growth on rich medium:*

Strain	Thin pili (EM)	Agglutinability by IgUB	Adh to HD	Adh to PS	ϕ Sensitivity			
					AP-3	ϕ1	ϕ11	215H
Rag-1	+	+	+++	+	S	R	S	S
MR481	–	–	–	–	S	R	S	S
RV-1	++	++	+	+	S	R	R	S
RV23-4	++	++	++	+	R	S	S	S

Results suggest the *following* model:

1) Pili or pili-associated component can mediate adherence to polystyrene and hydrocarbons.

Evidence

(a) When MR-481 finally comes upon HD in liquid, more than 90% of colonies adhere to PS. Random study of four of these clones – all grew back thin pili.

(b) Of all the various mutants in the lab, tested, only AB-15 showed adherence deficiency. Cells grown on EtOH did not adhere to HD nor PS.

Adherence of RAG-1 grown in complex medium is due to the presence of several components on the cell surface, one of which is thin pili.

Evidence:

a) MR-481 lacks at least five surface antigens as shown by C.I.E.

b) ~~AB-15~~ AB-15 is conditionally adherent – adheres following growth on complex medium but doesn't have pili.

c) The transition 481 → rv1 → rv234 is accompanied at each step by differences in adherence and phage sensitivity; ~~suggesting that~~ RV-1 has grown back thin pili; RV23.4 ~~has~~ still has thin pili but also other adherent properties.

3) Adherence important for growth on HD under limited emulsification.

Evidence:

(a) MR-481 does not grow on HD (J. Bact.)

(b) Following prolonged incubation and transfers on HD, MR-481 produced ~~too~~ two successive partial revertants exhibiting increased adherence to hexadecane.

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Adherence of Rag-1 strains grown on hexadecane plates

28/10/81

Rag-1, MR-481, RV-1 and RV23-4 were grown for three days on plates containing $\frac{PUM}{2}$ supplemented with hexadecane vapours. (All four strains grew similarly on these plates). The plates were scraped and cells were washed twice and suspended in PUM. Adherence to hexadecane and agglutination in presence of 5µl IgUB were carried out as usual.

1) Adherence:

Strain	Initial O.D.$_{400}$	% Adherence to HD (µl)		
		50	100	200
Rag-1	1.449	90	89	95
MR 481	1.401	4.5	7.0	14
RV-1	1.430	16	12	16
RV23-4	1.552	55	56	63

2) Agglutination: (500µl cells from previous expt. + 200µl PUM + 5µl IgUB)

Strain	Initial O.D.	$t_{1/2}$ (min)
Rag-1	1.023	~160
MR-481	1.003	> 900 (15h)
RV-1	1.005	60-65
RV23-4	1.251	60-65

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Adherence of E. coli to Hydrocarbons

26/6/80

Pathogenic *E. coli* strain 346 was harvested after 48 h. growth in brain - heart infusion broth/30°C and washed and resuspended in PUM to O.D.$_{400}$ = 1.603 . Adherence to hydrocarbon assayed as usual.

Controls (before and after mixing no difference) - 1.603
1.603

Results -

volume	Hex.	%	Oct.	%	Xyl.	%
10	1603	100	1596	100	1594	99
20	1603	100	1596	100	1550	97
50	1602	100	1587	99	1470	92
100	1606	100	1587	99	1429	89
200	1603	100	1594	99	1379	86

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Kobi and Mel 2/1/84

Procedure -

Cells of RAG-1 and MR-481 were grown O.N. (18h) in brain heart infusion broth, washed twice and resuspended to ~100 K.U. (A_{540}) in PUM. To 4.2 ml aqueous cell suspension were added 1 ml of hydrocarbon and the mixtures agitated on a Vortex. At various times phases were allowed to separate and the turbidity of the aqueous phase measured (K.U.; A_{540}). Results are presented below:

TIME (min)	RAG-1 + Hexadecane	RAG-1 + Octane	MR-481 + Hexadecane	MR-481 + Octane
0	98	98	98	98 ~~98~~ ~~88~~
1	19	0	~~87~~ 81	86 ~~82~~ ~~[illegible]~~
2	2	0	87	82 ~~91~~ ~~88~~
4	0	0	96	91 ~~97~~ ~~97~~
9	0	0	~~8~~94	97 ~~98~~ ~~96~~
39	5	0	100	98 ~~80~~
84	2	0	97	91
134	13	0	92	90
154	35	0	97	90

Conclusions -

1) Mutant MR-481 is non-adherent, even following prolonged shaking.

2) While initially RAG-1 cells were completely removed by the hexadecane, turbidity began to reappear following ~2 h. incubation. This signifies growth, emulsan production (release), or perhaps both.

3) RAG-1 cells were removed very quickly upon agitation with octane and remained attached to the octane droplets throughout the course of the expt. This indicates that in the absence of metabolism (growth), the cells remain bound to the hydrocarbon phase, and that the adherence is not reversed by prolonged agitation.

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Expt with Alex: Adherence to Epithelial Cells. 24/8/80

Rag-1 and 481-B (non-sticky mutant of RAG-1) were grown 18h. in brain heart infusion broth with shaking @ 30°. E. coli 346 and S. pyogenes M-5 were grown in the same medium 48h. at 37° without shaking. Cells washed in PBS and resuspended to 100-200 K.U. Adherence to epithe cells as usual (0.5 bacteria + 0.5 ml epithelial cells).

Results –

No	Strain	"W" Emulsan 100 μg/ml final	Mannose 2.5%	Glucose 2.5%	# cells scored	% Adh (net)	% of control
1	RAG-1	−	−	−	290	50	← 100
2	"	+	−	−	255	26	52
3	"	−	+	−	305	47	94
4	"	−	−	+	288	50	100
5	"	+	+	−	390	34	68
6	481 B	−	−	−	230	3.6	7.2
7	M-5	−	−	−	~~215~~	~~26~~	← 100
8	"	+	−	−	~~304~~	~~9.2~~	35
9	346	−	−	−	~~145~~	~~49~~	
−	control − bacteria				~~99~~	~~2/99~~	

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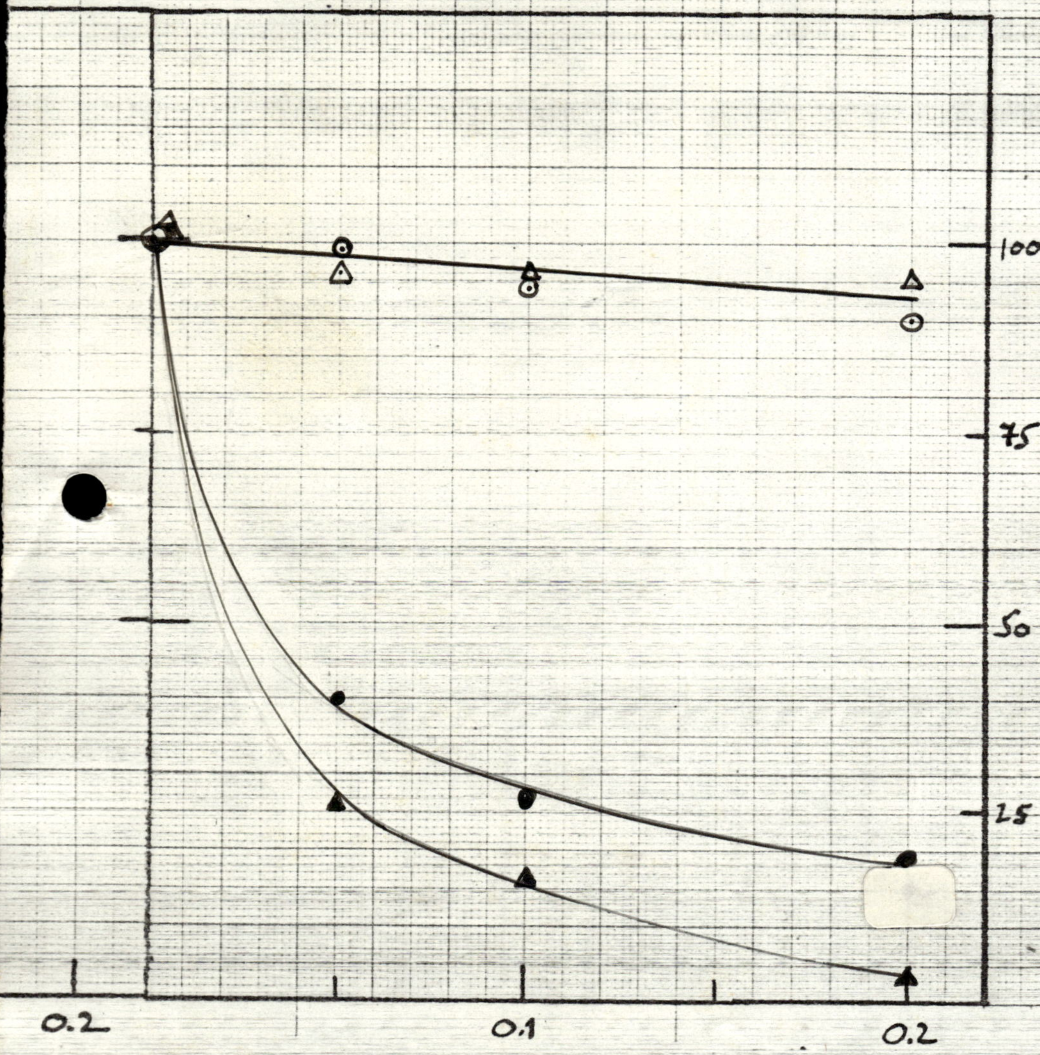
~~Fig. 31~~. 26

15 vs 92

481 vs RAG-1

2% ethanol

2/12/81
af



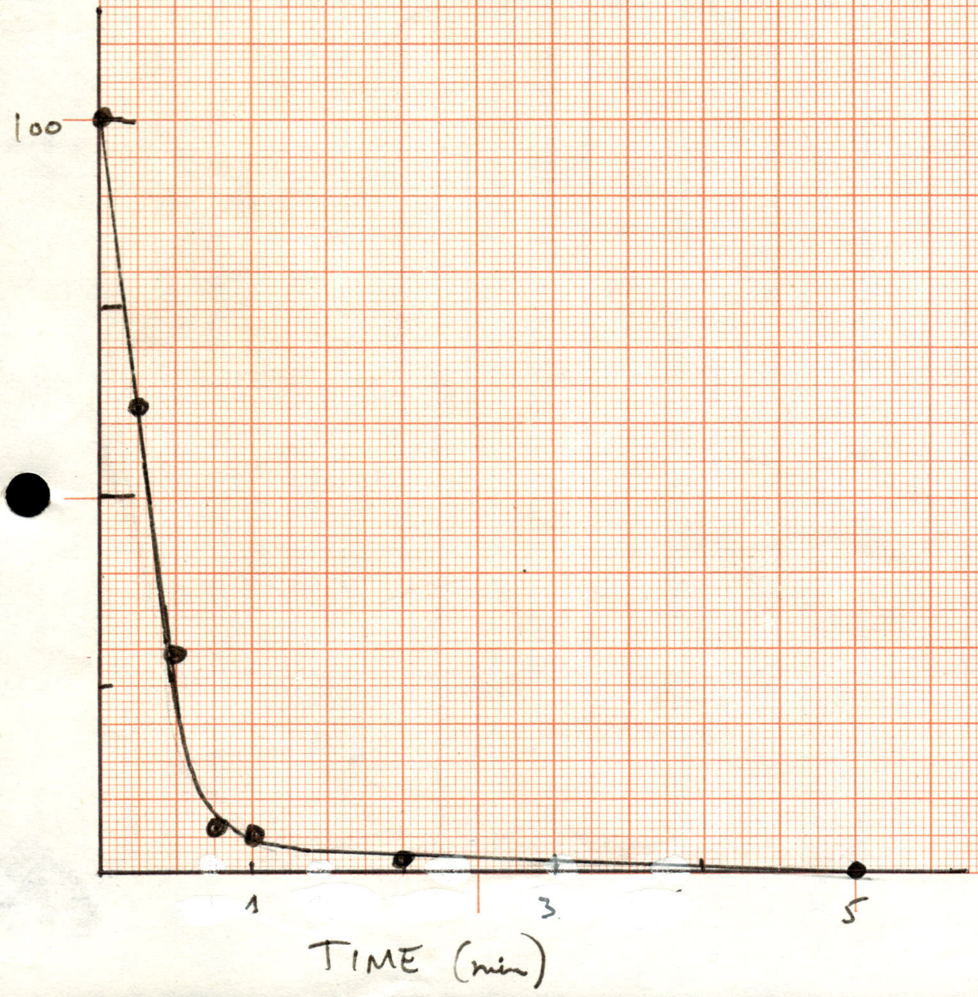
EtOH gram.

1.35-1.55

19/8/81

26/6/81

Fig. 10



Rag-1-92 and AB-15: growth on hexadecane vs acetate 2/11/81

Fig. 29

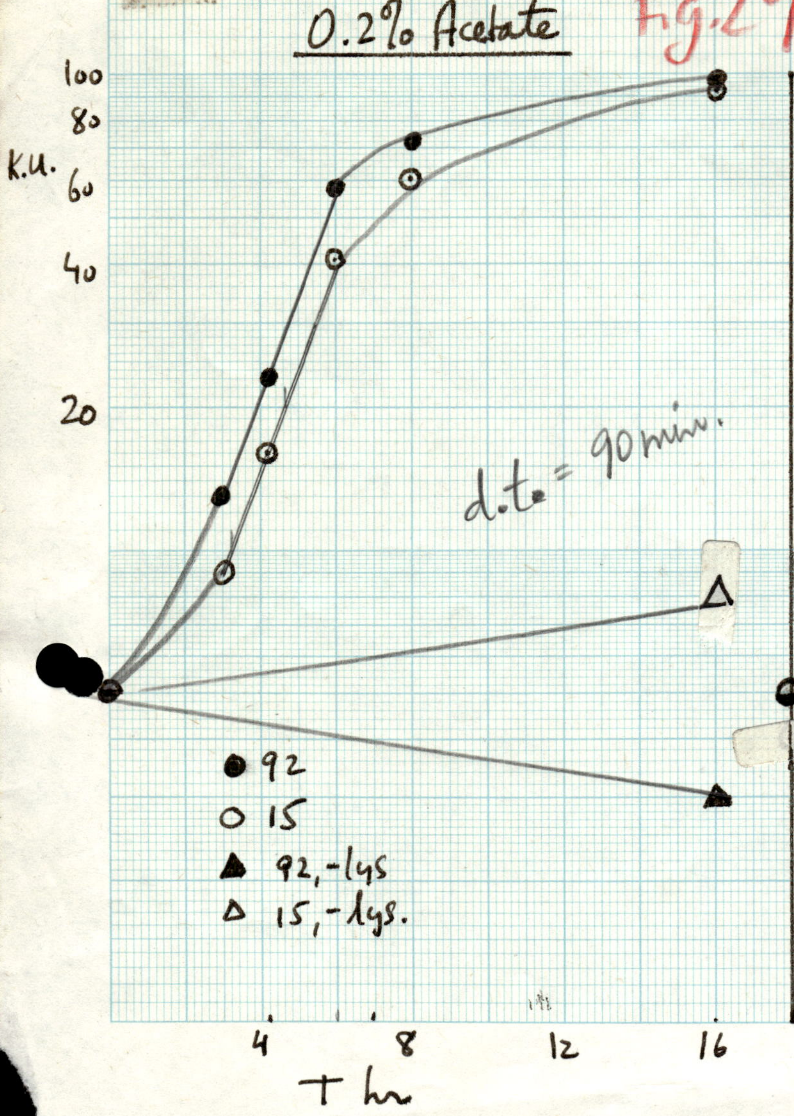
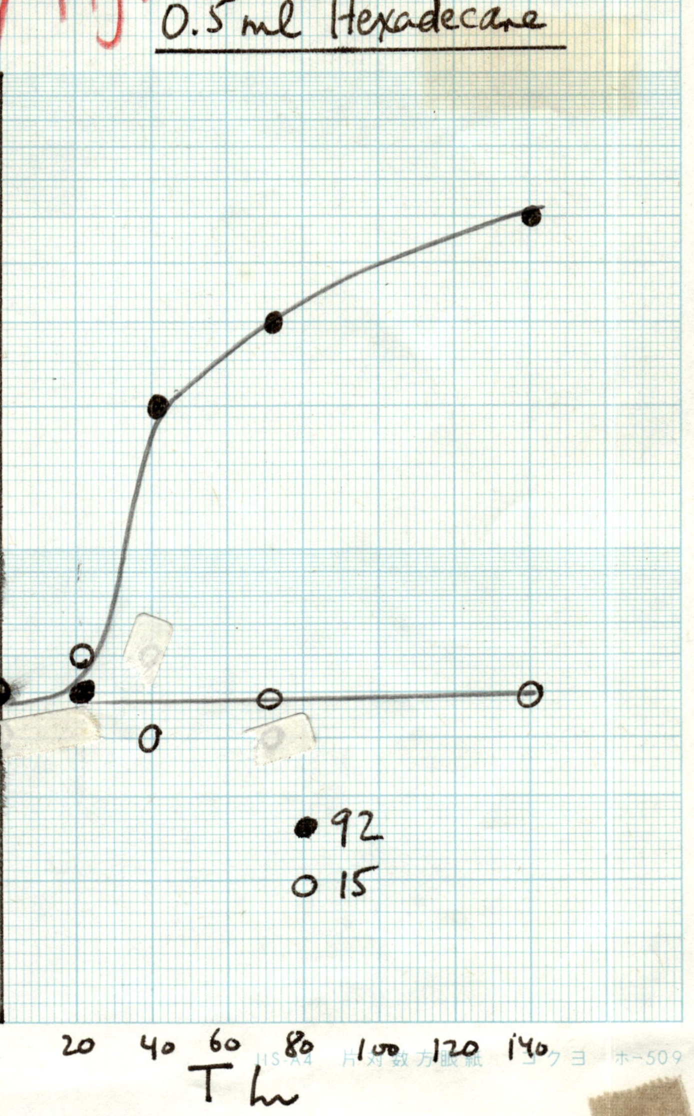
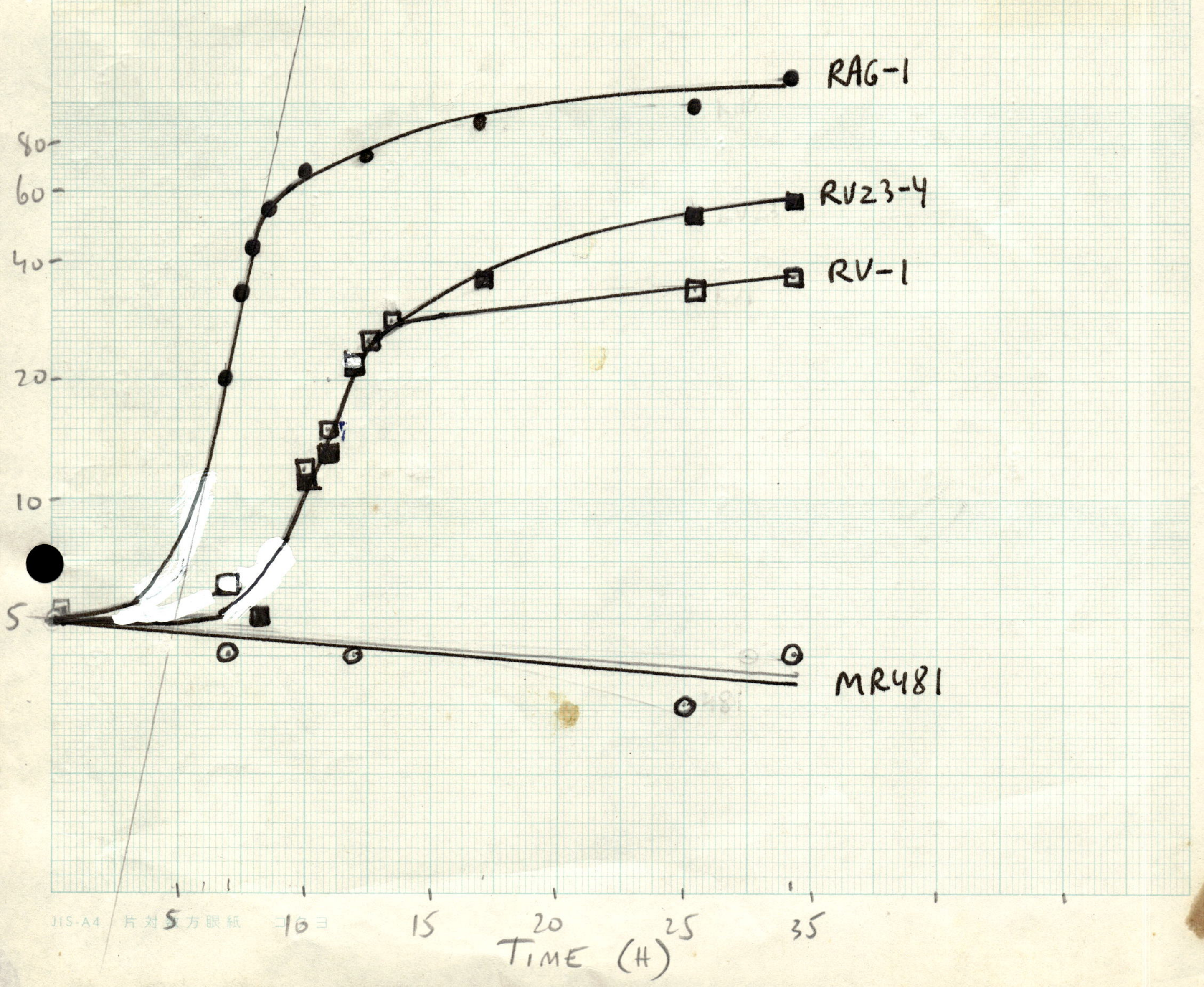


Fig 30




RAG-1
RV23-4
RV-1
MR481
80
60
40
20
10
5
5
10
15
20
25
35
TIME (H)

רג' : זה הגרף השוהרין
של התצגה.

MEL

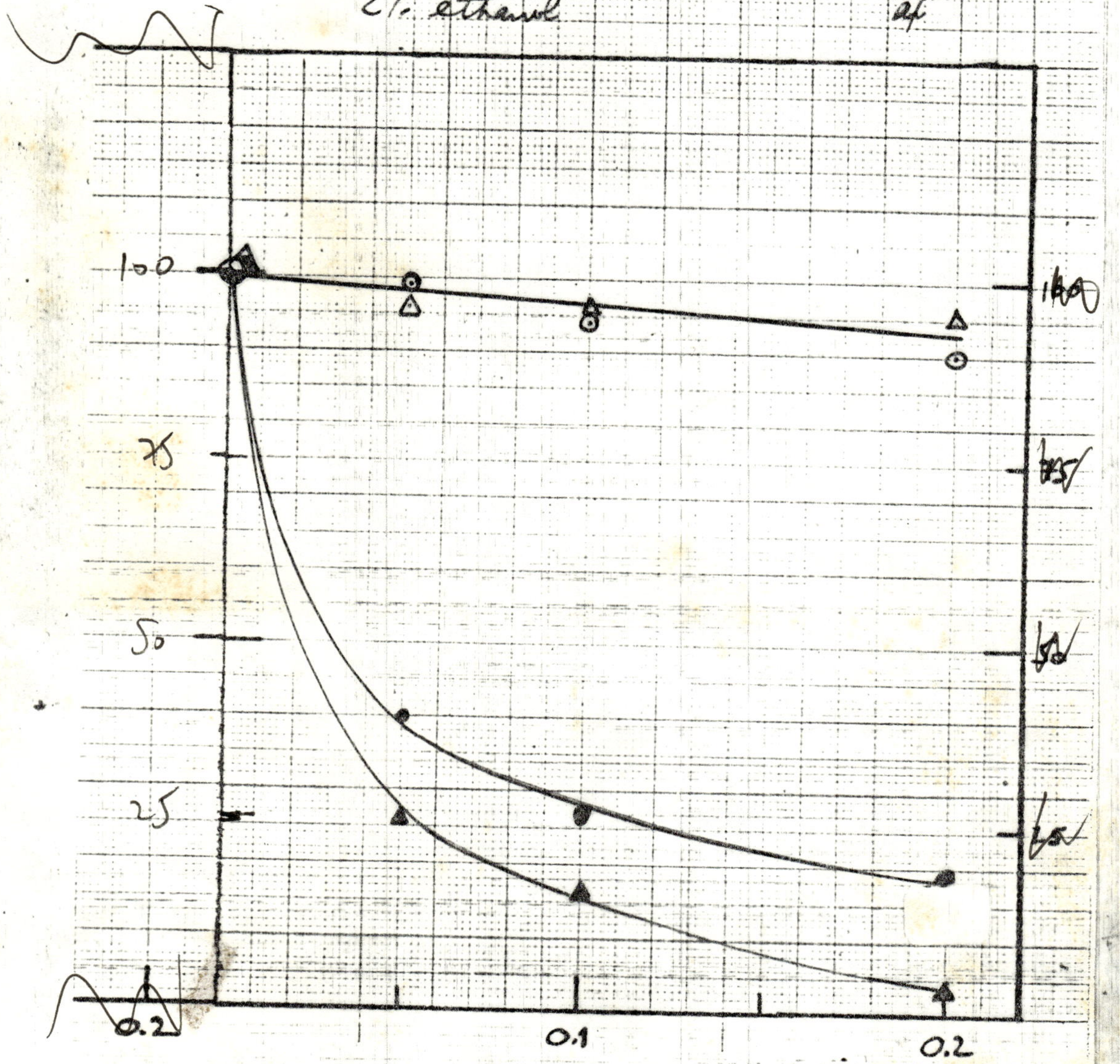
~~Fig. 31.~~ Fig. ~~30.~~ 28

15 vs 92

481 vs RAG-1

2% ethanol

2/12/81
af



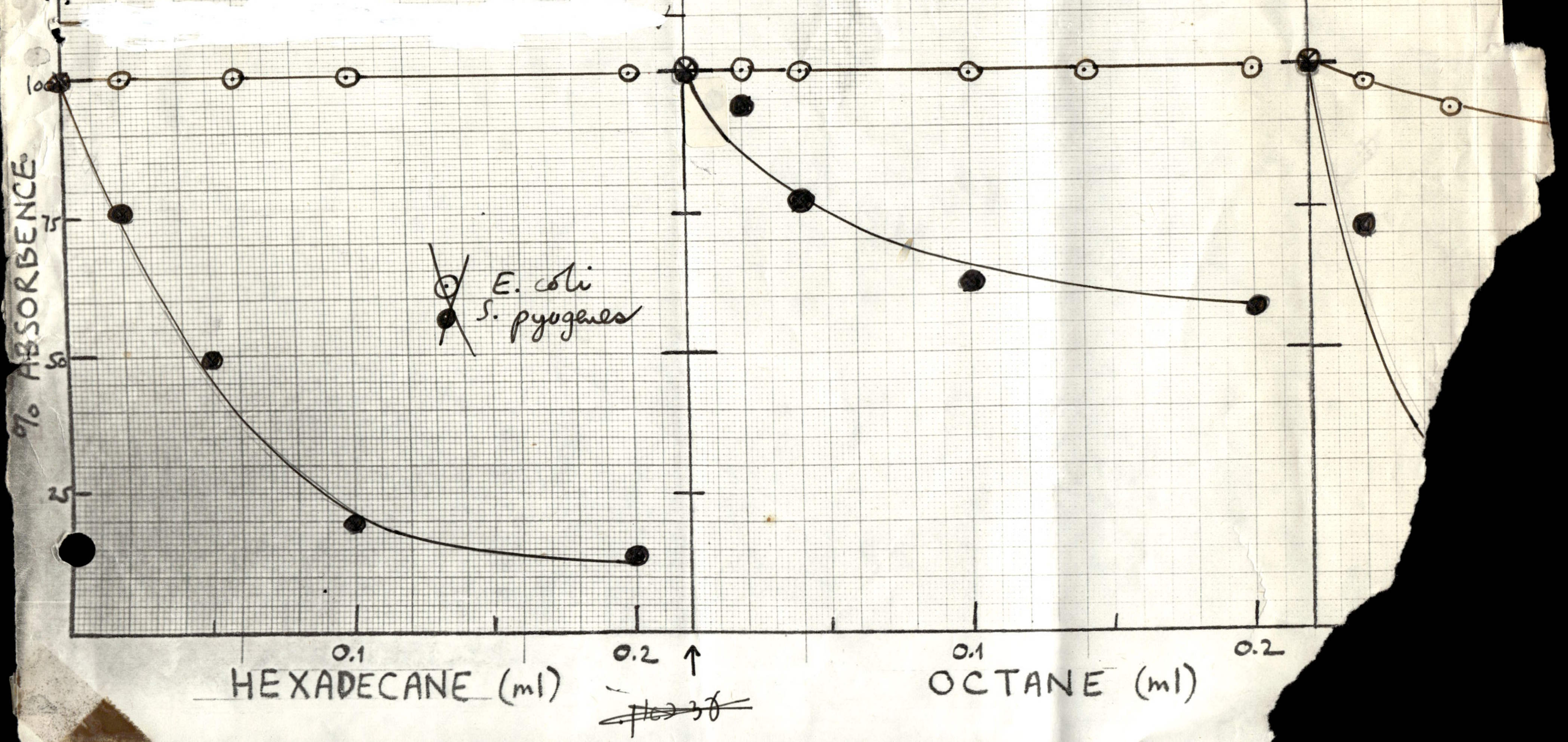
EtOH gram.

1.35–1.55

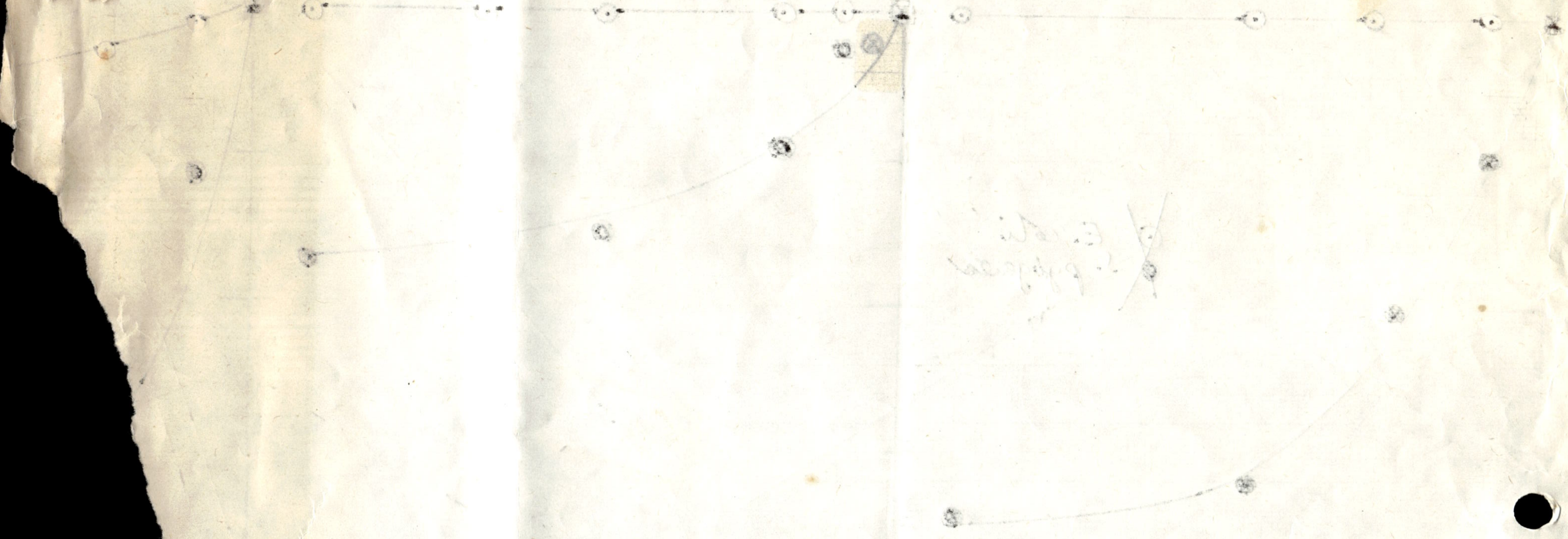
19/8/81

26/6/81

Fig. 16

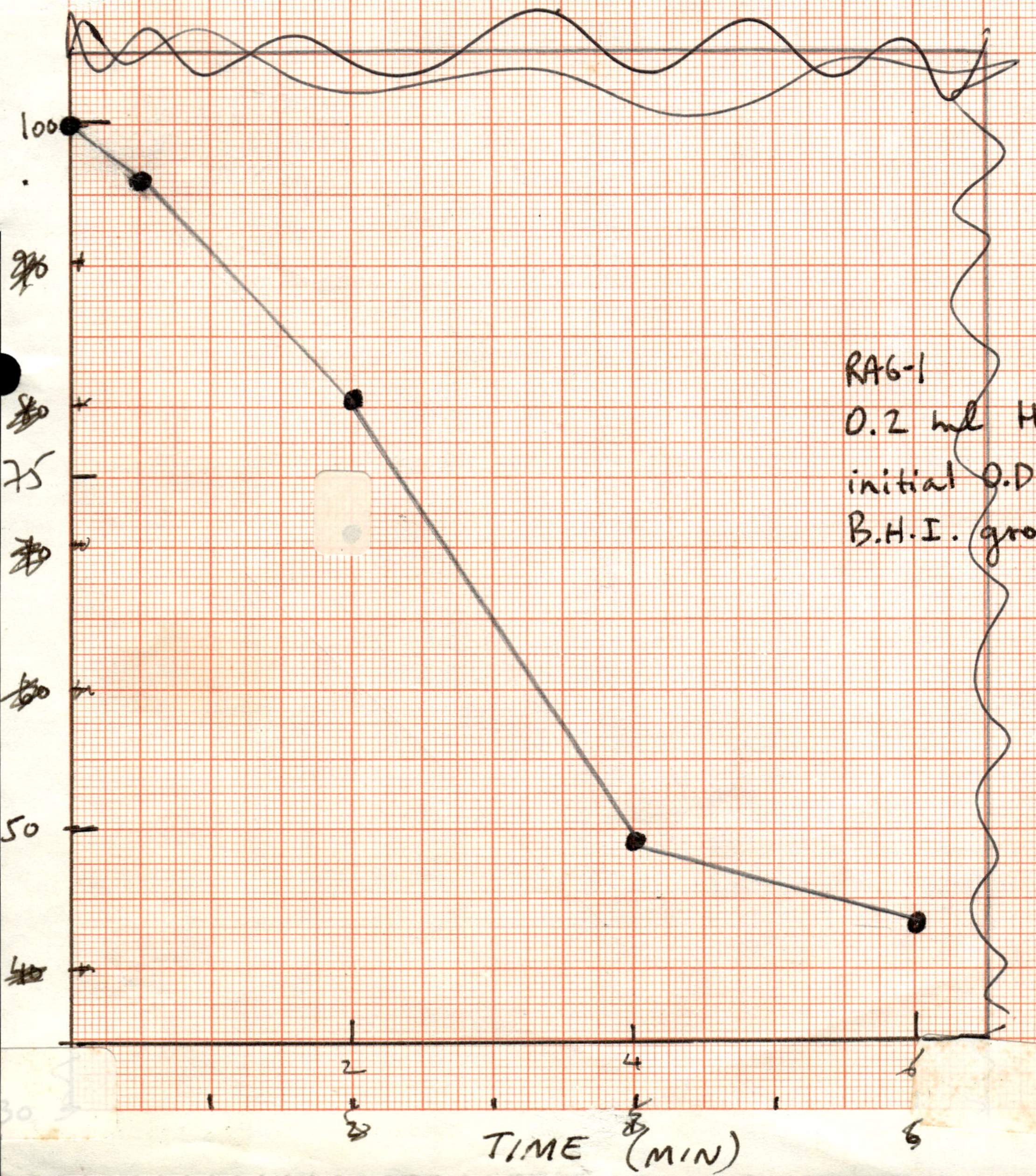

% ABSORBENCE
100
75
50
25
E. coli
S. pyogenes
0.1
0.2
HEXADECANE (ml)
0.1
0.2
OCTANE (ml)

1/6/80
26/



Adherence to Hexadecane of Sonified Cells 1/6/81

Fig. 21



RAG-1
0.2 ml HD
initial O.D. 1.20-1.35
B.H.I. grown

Release of RAG-1 cells by Freezing and thawing 22/1/81

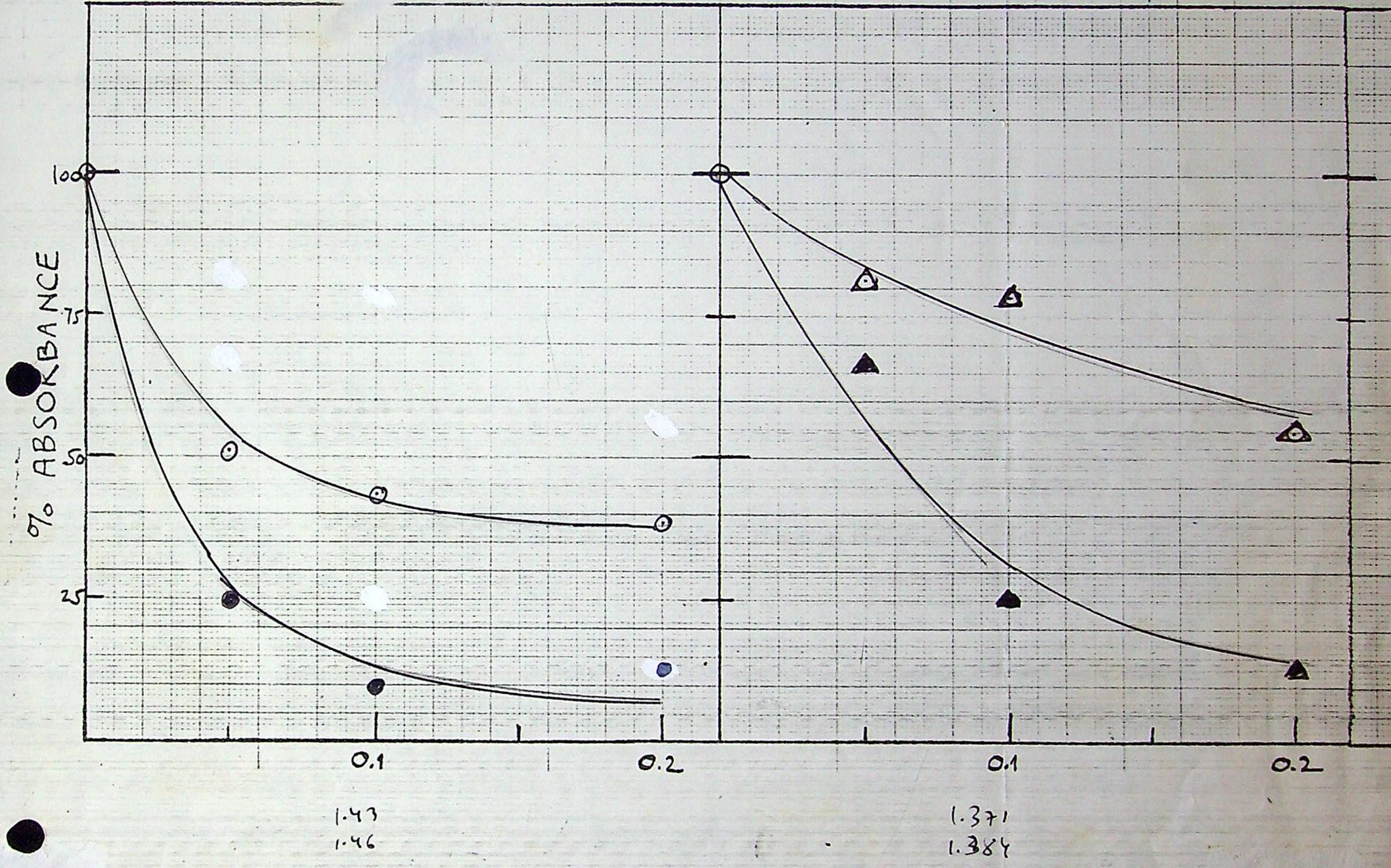
Cells grown 16h. in brain heart infusion, washed twice and resuspended sterilely in PUM in four test-tubes, 1 to 4. To test-tubes 1-3, 0.2 ml. hexadecane was added ~~to~~ and the mixtures vortexed as usual. Following phase separation, viable counts were taken of the lower aqueous phase. (Test-tube ~~four~~ #4 was vortexed in the absence of hd). The lower phase of test-tube 1 was removed and its turbidity measured. Temp. of Test-tubes 2 and 3 was reduced briefly to 12°C, causing the hexadecane to freeze. Upon thawing the hydrocarbon droplets coalesced and turbidity reappeared in the aqueous phase. Viable counts were taken and the turbidity of the lower aqueous phase was measured. Results are presented below.

T. T. #	HD	Following assay (adherence) A400	%*	c.f.u. x10^7	%	Following release A400	%	c.f.u. x10^7	%
1	+	0.097	8.1	2.56	5.7	–		–	–
2	+	–	–	1.25	2.8	~~9.6~~ 0.732	~~21~~ 61	9.6	21
3	+	–	–	1.36	3.0	~~8.3~~ 0.545	~~19~~ 45	8.3	19
4 (no hd)		1.204	–	45.0	–	–	–	–	–

The adherence assay left 2.8-5.7% of the cells (by c.f.u.) in the aqueous phase. After the release 19-21% of the c.f.u. and 45-61% of the turbidity could be found in the aqueous phase. Results indicate that ① the turbidity results are much higher (perhaps death, but more likely clumping ~~② cannot~~ be and ② the release is not quantitative enough for growth experiments unless improved.

* % of control

Fig. 20.



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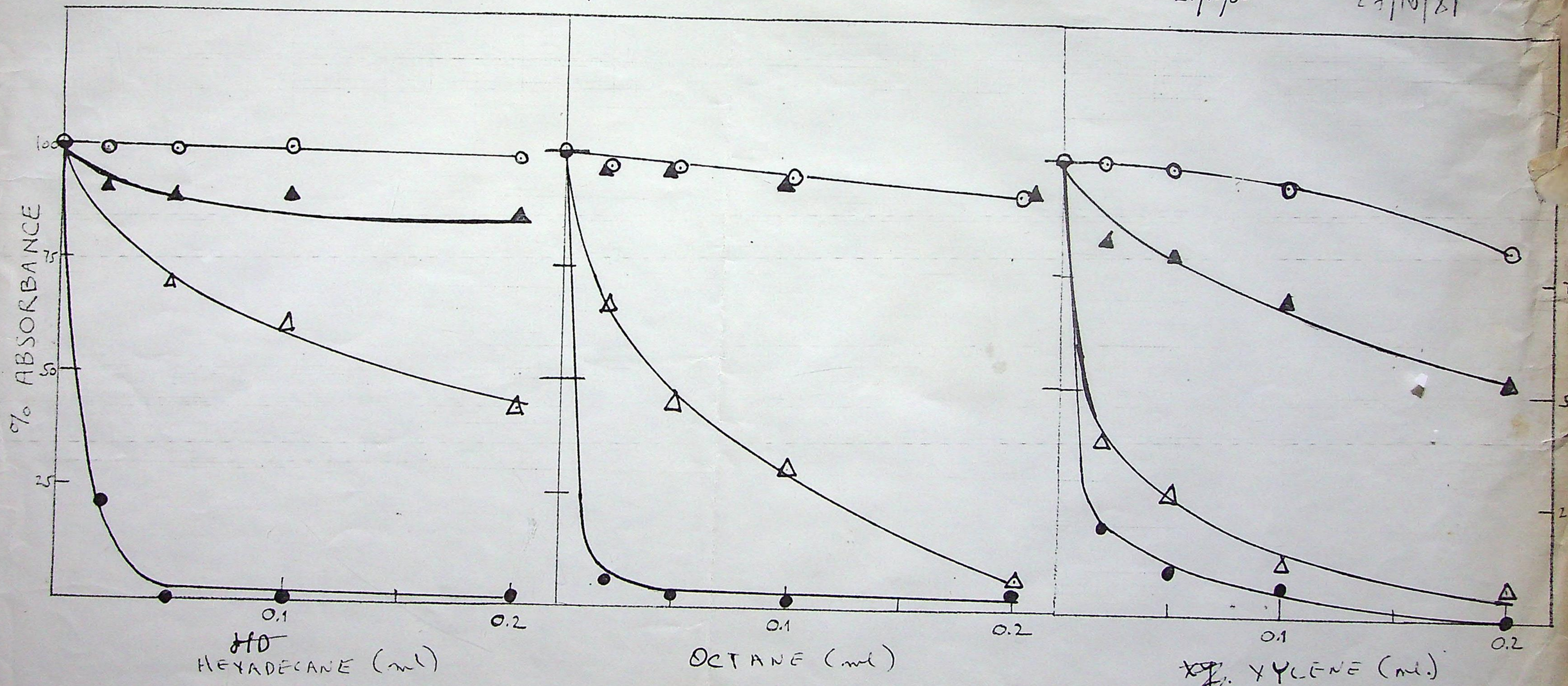
[illegible], MR-4[illegible], RV-[illegible], RV-2[illegible].

7.

22/7/80
and
29/10/80

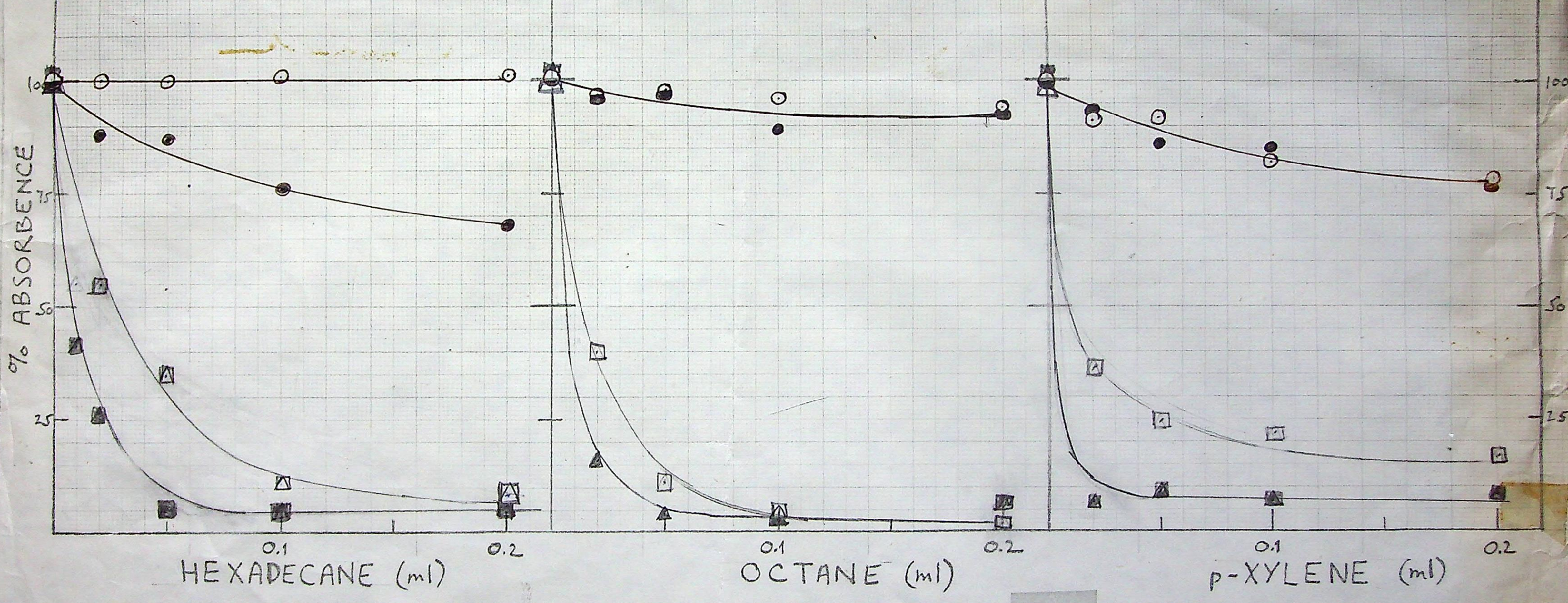
6/10/81
and
27/10/81

1



Rag-1	1.489	(●)
MR481:	1.463	(○)
: RV-1	1.556	(▲)
RV-23	1.461	(△)

1.46 to 1.56



1407
PAS ○ NB
279 ● 48h culture (HD).

1-48h RAG1 △ NB
▲ HD.

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Purpose

Decapsulation of A. calcoaceticus BD4 cells with a phage induced enzyme and determination of adherence properties of capsulated and decapsulated cells.

Methods

Microorganism and growth conditions

A. calcoaceticus BD4 originaly isolated by Taylor and Juni (   ) was a gift from K. Bryn. Strain BD4 was maintained on Nutrient Agar plates in the cold. In all experiments strain BD4 was grown in a glucose-minimal (GM) medium that contained per liter of deionized water: 5.0 g D-glucose, 9.17 g $K_2HPO_4 \cdot 3H_2O$, 3.0 g KH_2PO_4, 4.0 g $(NH_4)_2SO_4$ and 0.2 g $MgSO_4 \cdot 7H_2O$; final pH 7.0. For semi-solid media Bacto agar was added to GM medium to a final concentration of 1.6%, 0.6% or 0.3% to obtain ~~gm~~ GM agar, soft agar or "sloppy" agar, respectively.

Phage isolation

A sample of sewage water taken from the Tel-Aviv municipal sewage treatment plant was filtered through Whattman No 1 filter paper and then centrifuged at 8000 x g for 5 min at 4° C. Several drops of chloroform were added to the supernatant fluid of which 0.1 ml was then inoculated together with 0.5 ml of stationary BD4 cells (grown in GM medium) into a 100 ml flask containing 20 ml GM medium. The flask was incubated with shaking (150 rpm) at 30°c for 1 day and treated with several drops of chloroform. Appropiate dilutions were then plated by the soft agar overlay method ( ) together with stationary BDH cells onto GM agar plates. After incubation at 30°c for 2 days several plaque types appeared of which the dominant type had a small clear center surrounded by a large turbid halo, a characteristic typical of capsule degrading phages (, ,). Several such plaques were picked, resuspended in sterile GM medium and again plated as above. One isolate, termed φ SL-1 was chosen for further studies.

Isolation of crude capsule-degrading enzyme

Eight GM agar plates were each covered with 3 ml of "sloppy" GM agar containing 10^8 pfu/ml of φ SL-1 and $5 \cdot 10^8$ cells/ml of stationary phase BDH (grown in GM medium). The plates were incubated at 30°c over night untill total lysis occured. The "sloppy" top agar was then collected and the surface of the plates was washed with a total of 20 ml phosphate buffer (0.2 mM; pH 7.0). The wash was pooled with the top agar which was then centrifuged at 17,300 x g for 20 min at 4°c. To the clear supernatant (32 ml) 7.23 g of ammonium sulfate was added to reach 40% saturation. After leaving over night in the cold, the ammonium sulfate solution was centrifuged at 17,300 x g for 20 min, and the pellet fraction was resuspended in 4 ml of distilled water. The suspended pellet was then centrifuged in a Beckman model L3-50 ultracentrifuge at 140,000 ×g for 2 hr at 4°C. This latter procedure caused a precipitation of 99.5% of the plaque forming units and the resulting supernatant fraction was used as a crude capsule degrading enzyme preparation.

Enzymatic decapsulation of strain BD4

Strain BD4 cells were spread ~~over~~ with a sterile swab ~~d~~ over the entire surface of a GM agar plate. After incubation at 30°C for 3 days the cells were harvested from the surface of the plate, ~~and~~ were washed once and were resuspended in distilled water ~~and were resus~~ to a turbidity of 250 K.U. as determined in a Klett Summerson photometer (green filter). ~~0.2 ml of the~~ To 4.8 ml of the washed cell suspension, 0.2 ml of the crude capsule-degrading enzyme preparation was added and the mixture was incubated at 30°C. ~~Washe~~ Washed cells without enzyme and crude enzyme diluted 1:25 in distilled water were used as controls. At timed intervals 1 ml samples were removed and centrifuged in an Eppendorf table-top model 5414 centrifuge in the cold. The clear supernatant was saved and the pellet was resuspended in distilled water to the original volume. The ammount of rhamnose- ~~[illegible]~~ containing exopolysaccharide was determined by measuring the rhamnose content both in the supernatant fraction and on the resuspended cells with the cysteine-sulfuric acid method ( ) using L-rhamnose as a standard. After removing samples for determination of rhamnose, the a cell pellets were ~~[illegible]~~ washed once and resuspended in 5 ml ~~buff~~ of buffer and then assayed for adherence to hexadecane as described previously ( ).

Results

Experimental 1) see figures.

2) The average of the sum of capsular and extracellular rhamnose determined at all time points was 244 μg/ml (S.D. = 7.1 μg/ml).

Controls 1) cells only - capsular rhamnose - 93% - 97% and extracellular rhamnose - 3% - 7% at all time points.

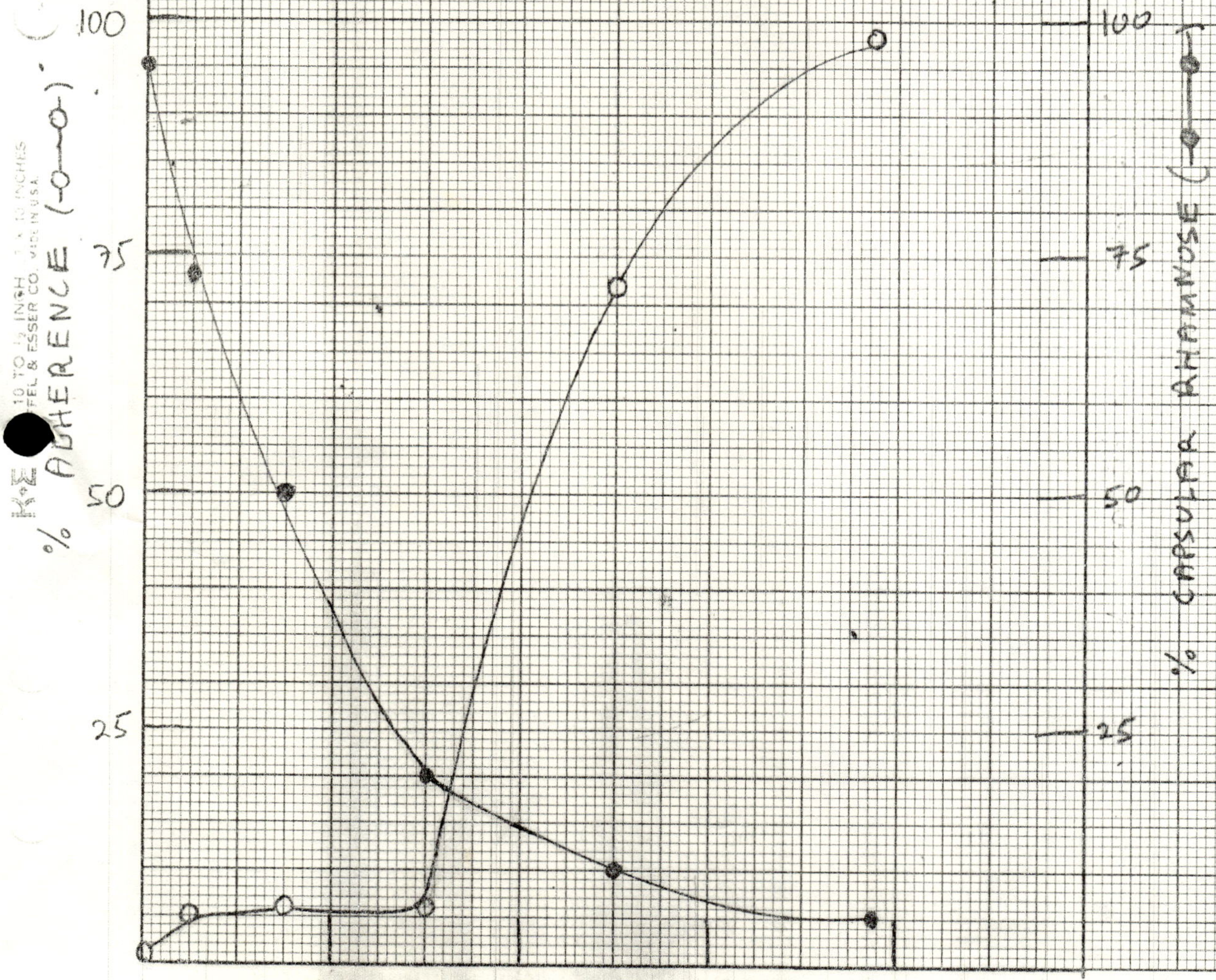
2) cells only - 0% - 2% adherence at all time points.

3) Total counts of control and experimental at times 0 and 15.5 hr gave the same value of 9×10^8 cells/ml.

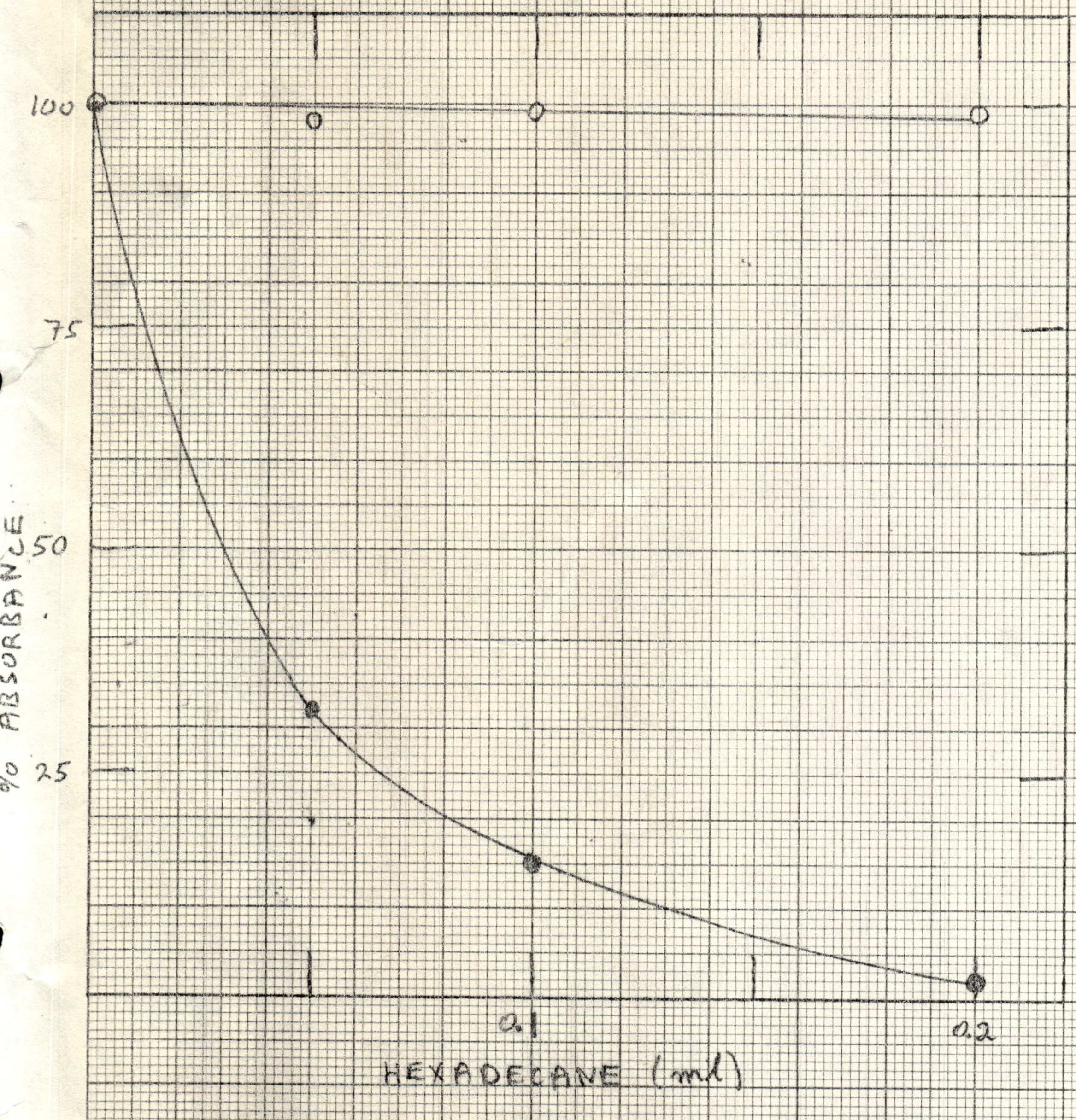
4) Enzyme only - The rhamnose content was less than 0.2 μg/ml.

Fig . Kinetics of enzymatic decapsulation and subsequent adherence properties of *A. calcoaceticus* BDH. Strain BDH cells were incubated at 30°C with φ SL-1 capsule-degrading enzyme and samples were assayed for rhamnose content and adherence to hexadecane (200 µl) as described in Material and Methods. Capsular rhamnose is presented as the percent of the total capsular and extracellular rhamnose (244 µg/ml); adherence is presented as the percent of cells removed from the aqueous to the hexadecane phase in the adherence assay as measured by the decrease of optical density at 400 nm.

Fig . Adherence of enzymatically decapsulated *A. calcoaceticus* BDH to hexadecane. Samples taken after 15.5 hr of incubation with φ SL-1 enzyme as described in Fig __ were assayed for adherence to ~~[illegible]~~ different volumes of hexadecane. The results are plotted as the percent decrease in absorbance (400 nm) in the aqueous phase of the adherence assay of cell samples ~~incubated~~ incubated in the presence (●) or absence (○) of enzyme.



K+E 10 TO 1/2 INCH 7 X 10 INCHES
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 1320



K+E 10 TO ½ INCH 7 X 10 INCHES
FEL & ESSER CO. MADE IN U.S.A.
46 1320

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26/4/81

To 9:55	10:03	10:10	1020
R lg	1779		
R ◡	1671		
R	1735		
92	1381		
	1354		
	1360		
1	1343	1323	1282
	1337	1330	1289
	1366	1362	1355
23	1304	1282	1236
	1269	1240	1209
	1416	1398	1382
B	1451		
	1418		
	1425		
15	1292		
	1255		
	1268		
To 10:15		10:20	
R		1289	
		1253	
		1297	

1035	11:00	1120	1150
1675	1522		
1593	1521		
1671	1650		
1290	1227		
1289	1244		
1317	1294		
1231 ~~1240~~	1097	984	801
1261	1214	1187	1150
1337	1315	1305	1282
1178	1125	1073	962
1190	1171	1137	1112
1361	1341	1319	1289
1410	1393		
1376	1354		
1391	1370		
	1229		
	1198		
	1220		
1040	1105		
1256	1196		
1198	1188		
1267	1243		

1218	1248	1:20
468	419	323
1479	1446	1383
1599	1607	1589
522	452	328
1138	1093	1016
1226	1211	1189
622	536	455
1119	1096	1057
1266	1247	1230
915	867	762
1095	1084	1051
1281	1276	1248
1344	1336	1316
1301	1291	1271
1319	1306	1286
1195	1180	1164
1156	1144	1126
1185	1178	1161
1252		
787	555	514
1141	1123	1099
1200	1200	1193

50 µl 1:10 + 700 Pum.

	from bottom 2:05	2:45	3:45	4:30 PM	9:50 A.M.	
R Ig	.279	246	189	149	.048	
⊃	1041	987	913	827	.246	
R	1167	1165	1154	1139	.980	
92	258	228	183	154	.056	
	918	797	644	537	.179	
	1168	1161	1143	1126	.999	
1	351	315	274	219	~~0.109~~	.074
	1002	976	914	861	~~1.103~~	.459
	1200	1192	1171	1213	~~1.065~~	1.041
23	686	618	551	508	.231	
	1020	995	949	904	.560	
	1227	1226	1206	1188	1.057	
15	1142	1141	1118	1112	847	
	1110	1112	1092	1089	886	
	1143	1145	1128	1117	945	
B	1296	1291	1274	1249	1.090	
	1250	1247	1228	1207	1.103	
	1265	1260	1241	1218	1.065	

26/11/81

Aggl. of cells from freezer, washed down and suspended in PUM to O.D. 1.25-1.45. 50 µl of IgUB and [illegible] antibody added.

To = 955

R Ig R ⊃ R 1 Ig 1 ⊃ 1 23 Ig 23 ⊃ 23 B Ig B ⊃ B 15 Ig 15 ⊃ 15

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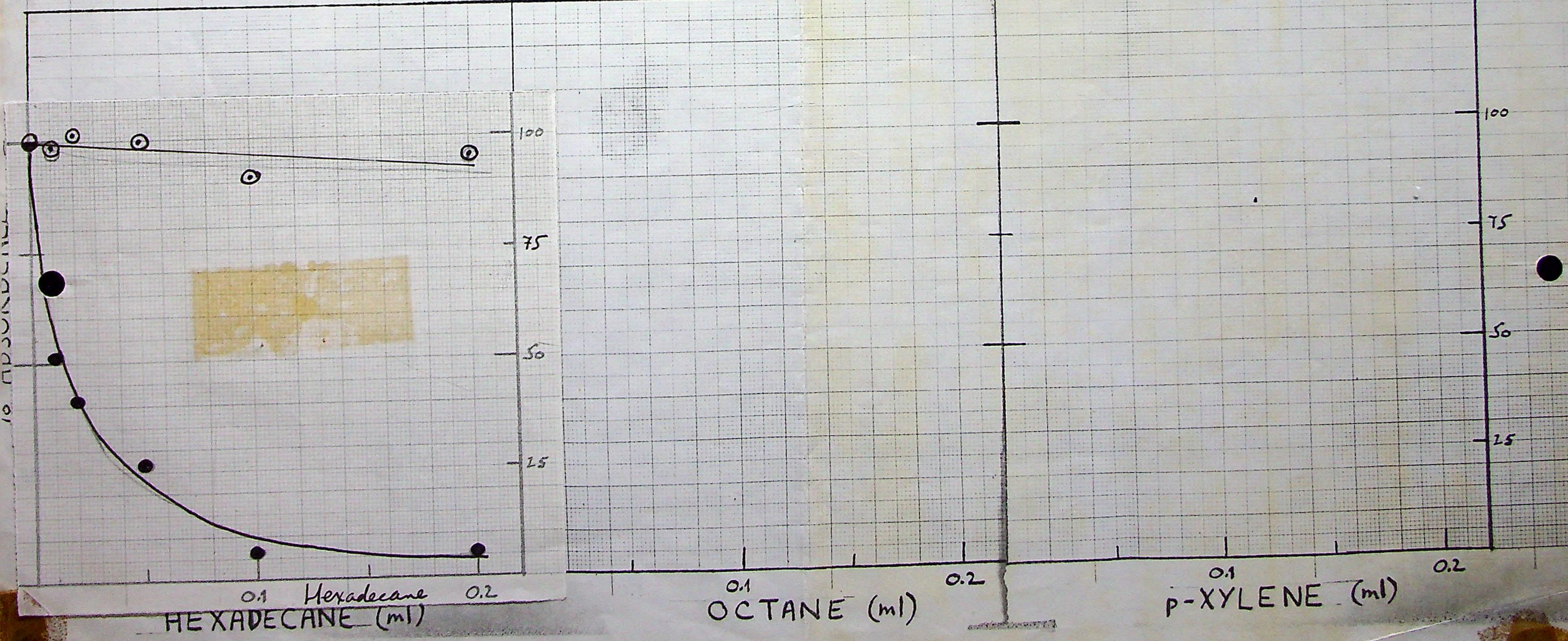
835 גל

([illegible])



100
75
50
25
0.1
0.2
Hexadecane
HEXADECANE (ml)
0.1
0.2
OCTANE (ml)
100
75
50
25
0.1
0.2
p-XYLENE (ml)

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J. Bac. 1976, 127, 481, Scott, Makula + Fin.

Hydrocarbon Inclusions

grew cells of Acinetobacter on yeast extract and hexadecane.

y.e – 2 membrane fractions, presumably cytoplasmic membrane and outer membrane. hexadecane grown bugs produced, in addition, another membrane fraction rich in lipids, w/low density, containing hexadecane. They "identify" these vesicles (0.2 μm diameter) with hydrocarbon inclusions seen in whole, cells.

interesting refs.

4. Eur J. Biochem – lipoprotein binding outer membrane to peptidoglycan
1969, 10, 426-438

✓ 5. 6. J. Gen. Microb. Fine structure of methane and
1970, 61, 227-232 other hydrocarbon-utilizing bacterium.

10. Topography of bacterial cell wall:
1969 Annu. Rev. Microbiol. 23, 159-198.

19. Makula + Finnerty: 1970, J Bac 103, 348-355

21. " + Lockwood J. Bac 121, 250-258

27-28 J. Bac 1976 Scott & Finnerty, characterizing hydcbn inclusions

J Bac 127, p481-489 Scott + Fin. 1975

✓ look up McLee + Davis, Can J. Microbiol 18, 315-319.

✓ look up ~~Kl~~ Kling + Markovetz.

Arch. Microbiol 75, 83 Kennedy et al.

GLC cell pellets washed 5 x w H_2O
extracted w/ increasing ethanol concentration
+ analyzed by GLC.
cells grown on ~~pentane~~ C_{15} — ~~pentane~~ C_i etc etc.

X-ray diffraction S'jɔ.

Scott and Finnerty J Bac 128 p 481

Composition of hexadecane inclusions / mg protein

$C_{16}H_{34}$ 20.0 μmole

lipid-phosphorus 1

2 keto 3 deoxyoctulosonic acid 95 pmole.

7 16
12
192
33
226

226 mg/ml → 1 M
226 μg/ml ← 1 mM
4 μg / mg protein ← 20 μM

~~192~~ mg/l → 1 mM
192 μg/ml → 1 mM

✓ look up Shively Ann Rev Microb 28, 167-187.